A THESIS

entitled

SYNTHESIS AND REACTIONS OF DIHYDROXYALKYLCOBALAMINS; A MODEL FOR VITAMIN B_{12}-DEPENDENT PROPANEDIOL DEHYDRATASE

by

Ruth Mary Dixon
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To my parents

Heather and Hal Dixon

O Lord, our Lord, how majestic is your
name in all the earth!

You have set your glory above the heavens.
From the lips of children and infants
you have ordained praise
because of your enemies,
to silence the foe and the avenger.

When I consider your heavens,
the work of your fingers,
the moon and the stars,
which you have set in place,
what is man that you are mindful of him,
the son of man that you care for him?
You made him a little lower than the
heavenly beings
and crowned him with glory and honour.

You made him ruler over the works of your hands;
you put everything under his feet:
all flocks and herds, and the beasts of the field,
the birds of the air, and the fish of the sea,
all that swim the paths of the sea.

O Lord, our Lord how majestic is your
name in all the earth!

Psalm 8, NIV

Abstract

A model for vitamin-B_{12}-dependent diol dehydratase, 4,5-dihydroxypentylcobaloxime, was reported by Golding *et al.*[31,56]. This modelled the regiospecific abstraction of a hydrogen atom from C1 of a 1,2-diol by an alkyl radical in the diol dehydratase reaction, as it gave pentanal when photolysed under acidic conditions. In the present work, the same system was studied using cobalamins.

A series of dihydroxyalkylcobalamins (length of alkyl chain = 3 - 6 carbon atoms) was prepared. Alkylcobalamins were synthesised from hydroxo- or cyanocobalamin, which was reduced to cobalamin(I) using sodium borohydride or zinc dust, and treated with the appropriate alkyl halide or tosylate.

Synthetic routes to chirally pure or racemic alkylating agents are reported in Chapter 2. An original synthesis of 4-(3-iodo-2,2-dimethylpropyl)-2,2-dimethyl-1,3-dioxolan, starting from allyl alcohol and 2-methylpropanal, was developed, although the compound was not isolated pure, for reasons discussed in Chapter 2.

Alkylcobalamins were characterised by t.l.c., IR, UV, and ^{1}H NMR spectroscopy. High field NMR spectroscopy was found to be the most effective technique. The purity of alkylcobalamins could be assessed, and, furthermore, diastereoisomeric alkylcobalamins (differing only in the chiral centre on the alkyl ligand) could be distinguished. The methine protons on the corrin ligand of three cobalamins were assigned by n.O.e. difference spectroscopy. The chemical shifts and coupling constants of a number of alkylcobalamins are reported, and the structural information is discussed (Chapter 4).

Hitherto, the crystal structure of only one alkylcobalamin, the coenzyme, adenosylcobalamin, had been reported[33]. In order to ascertain whether the bond lengths and angles in the coenzyme were typical or atypical, the crystal structures of (*R*)- and (*S*)-2,3-dihydroxypropylcobalamin were determined (Chapter 5). The cobalt-carbon bond lengths do not differ significantly from that of the coenzyme (*R*, 2.00(2) Å; *S*, 2.08(3) Å; coenzyme 2.03(2) Å), but the Co-C-C angles are both smaller than that of the coenzyme (*R*, 119.6 ± 1.7 °; *S*, 113.6 ± 2.1 °; coenzyme; 125 ± 3 °). It seems that steric strain is relieved by an increase in the Co-C-C angle rather than in the Co-C bond length.

The thermolytic and photolytic behaviour of the dihydroxyalkylcobalamins was investigated (Chapter 6). No evidence of the rearrangement of any *vic*-diol to an aldehyde was seen, but instead β-elimination of hydrogen led to hydridocobalamin and an olefin, in most cases. Evidence of readdition of cobalt to the olefin, and further elimination to give a rearranged olefin, was found. The various reaction pathways available to dihydroxyalkylcobalamins are discussed.

These findings provide indirect support for the hypothesis that the cobalt atom does not participate in the rearrangement step of the diol dehydratase reaction. Instead it acts as a source of alkyl radicals, which are generated by the homolysis of the (relatively weak) cobalt-carbon bond in the coenzyme.

PUBLICATIONS

1. Synthesis and Characterisation by ^{1}H N.M.R. Spectroscopy of Diastereoisomeric Hydroxy- and Dihydroxy-alkylcobalamins.
   Ruth M. Dixon, Bernard T. Golding, Oliver W. Howarth and James L. Murphy, *J. Chem. Soc., Chem. Commun.*, 1983, 243.

2. The Crystal Structures of (*R*)- and (*S*)-2,3-Dihydroxypropylcobalamin; Comparison with the Structure of Adenosylcobalamin.
   Nathaniel W. Alcock, Ruth M. Dixon and Bernard T. Golding, submitted to *J. Chem. Soc., Chem. Commun.*

3. Concerning the Intermediacy of Organic Radicals in Vitamin-B_{12}-dependent Enzymic Reactions.
   Ruth M. Dixon, Bernard T. Golding, Samson Mwesigye-Kibende and D.N. Ramakrishna Rao, *Philosophical Transactions of the Royal Society, Series B*, accepted for publication.

CONTENTS

Acknowledgements

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DECLARATION

The work herein described was performed in the Department of Chemistry and Molecular Sciences, the University of Warwick, and the Department of Organic Chemistry, the University of Newcastle upon Tyne, during the period October 1981 to December 1984.

This work is thought to be original, and where other work is quoted, this is made clear and due acknowledgements are accorded. The work herein described has not been submitted for any other degree.

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SCHEMES

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FIGURES & TABLES

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ABBREVIATIONS

Å	Ångstrom unit
Ac	acetyl
Ado	5'-deoxy-5'-adenosyl
ATP	adenosine triphosphate
B	base
Cbl	cobalamin
CoA	coenzyme A
CTP	cytosine triphosphate
DH	dimethylglyoxime
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DNP	dinitrophenylhydrazine
ESR	electron spin resonance
Et	ethyl
FAD	flavine adenine dinucleotide
FID	free induction decay
FMN	flavine mononucleotide
FT	Fourier transform
GC	gas chromatography
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HPLC	high performance liquid chromatography
IR	infrared
mcpba	*meta*-chloroperbenzoic acid
Me	methyl
m.s., MS	mass spectrometry
NADH	nicotine adenine dinucleotide
NMR	nuclear magnetic resonance
n.O.e	nuclear Overhauser effect

OTf	triflate
Ph	phenyl
Pr	propyl
py	pyridine
R	alkyl
R_f	relative flow
SALEN	*N,N*-disalicylidene-*O*-ethylenediamine
SALOPH	*N,N*-disalicylidene-*O*-phenylenediamine
THF	tetrohydrofuran
t.l.c.	thin layer chromatography
Ts	tosyl
UV	ultraviolet

CHAPTER 1

INTRODUCTION

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INTRODUCTION

1.1 Vitamin B_{12} in nature

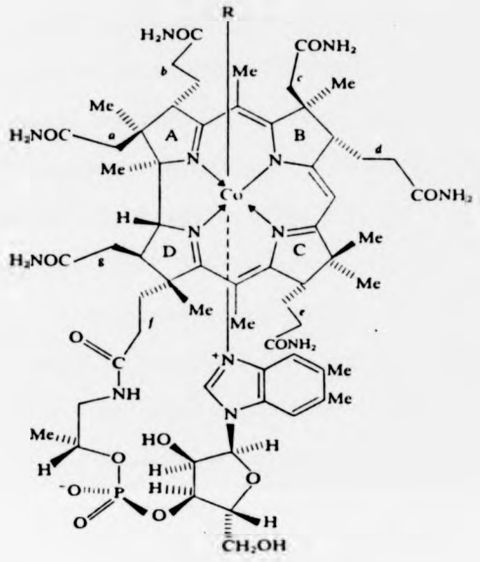
There is less than 5 mg of vitamin B_{12} in a normal human body, and yet a deficiency leads to the once fatal disease, pernicious anaemia, characterised by an inflamed tongue, lack of hydrochloric acid in the stomach, abnormal red and white blood cells, and neural degeneration. Injection of less than 10 μg of the vitamin results in an immediate feeling of well-being in the sufferer, followed by a rapid return to normality of the various symptoms. The mode of action of such a substance has therefore been the subject of much study since its discovery, the more so when it was found to be involved in the catalysis of previously unknown reactions, which were then without parallel in organic chemistry.

The active forms of vitamin B_{12} are methyl- and adenosylcobalamin (Figure 1.1). They are the only naturally occurring organometallic compounds so far discovered with biological functions.

Two, or perhaps three, vitamin-B_{12}-dependent reactions have been identified in humans and other mammals. They are methylmalonyl-CoA CoA-carbonyl mutase[1] (E.C.5.4.99.2), (possibly) leucine-2,3-aminomutase[2] (E.C.5.4.3._) and 5-methyl-H_4-folate homocysteine methyltransferase[3] (E.C.2.1.1.13). The first two are adenosylcobalamin-dependent enzymes found in mitochondria, and the third is a cytoplasmic enzyme which uses cobalamin as a methyl carrier. It is not yet clear, however, how deficiencies in these enzymic reactions lead to the symptoms of pernicious anaemia.

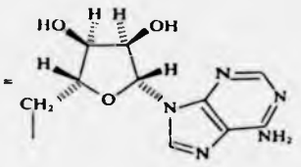
Vitamin B_{12}, in the form of hydroxo- or cyanocobalamin, is required by humans in very small amounts (about 1 μg per day to

Figure 1.1



R = CN
R = OH
R = Adenosyl
R = Me

Adenosyl =



supplement the body's reserves of 2 - 5 mg). Cobalamins are synthesised only by microorganisms (certain bacteria and protista), fungi and algae[4,5], and are required by all mammals and higher organisms, although probably not by plants. Reports claiming the presence of cobalamins in plant material have been disputed[5], as the small amounts found could usually be attributed to insect and microorganic remains. A vitamin-B_{12}-dependent enzyme has been reported[6] in plant tissue, but other workers have not confirmed these results. The vitamin is found in meat, particularly in liver, and in dairy products, so certain diets consisting entirely of vegetable products can lead to deficiency. In addition, about 0.1 % of the population suffers from pernicious anaemia[7], an inherited disease which prevents the effective uptake of vitamin B_{12} in the intestine, and they require periodic injections of cyano- or (preferably) hydroxocobalamin. Mammals are able to convert these forms of vitamin B_{12} into the coenzyme forms, but there is some evidence that hydroxocobalamin is converted more efficiently[45]. Increased vitamin B_{12} requirements (*e.g.* neoplastic disease, pregnancy) can also lead to deficiency. The cobalamin levels in the liver (where most vitamin B_{12} is stored) and other tissues begin to decrease long before the plasma cobalamin is decreased, and so a deficiency may be present for years before it is detectable in the blood. Assays based on the ratios of the various cobalamins can, however, give an indication that a deficiency is developing.[45]

The distribution[8,45] of naturally occurring cobalamins in various tissues shows that adenosylcobalamin is the most abundant form in all solid tissues, with various amounts of methyl- and hydroxocobalamin. It is also the most abundant in bone marrow and

in blood cells, while methylcobalamin is more abundant in plasma and in milk. Cyanocobalamin is probably only found in the blood of smokers, formed by the reaction of other cobalamins with inhaled HCN, and in certain pathological conditions.[45] The vitamin was first isolated as cyanocobalamin, and this was thought to be the form in which it exists in the body. In fact, cyanocobalamin was produced by reaction of the naturally occurring forms with light and with the cyanide in charcoal columns used in the isolation procedure.

Ruminant animals get sufficient vitamin B_{12} from that synthesised by bacteria in the fore-stomach, but deficiencies can occur if their diet lacks cobalt. Non-ruminant animals may absorb some of that synthesised in the gut, but gain most of what they need from the bacteria, fungi and yeasts growing on droppings on the pasture. Animals raised on purified vegetable diets were found not to grow at the usual rate, and hens laid eggs which failed to hatch, until animal protein was added to their diets. This corrected these conditions, and allowed much more intensive farming methods to be practised. The search for the 'animal-protein factor' showed that it was produced by microorganisms and fungi, as well as being found in animal products, and it was eventually found to be identical to the 'anti-pernicious-anaemia factor' (that is, vitamin B_{12}), found in liver.[5] Fermentations gave a much cheaper source of vitamin B_{12} than the extraction of large amounts of beef liver.[9]

A wide range of naturally occurring corrinoids is found among the bacteria[8], differing in the nucleotide base and the side chains, as well as in the axial ligands. Mammalian enzymes, however, accept only cobalamins, so the present work is concerned with these.

1.2 Discovery and structure elucidation

1.2.1 Discovery

In 1824 Combe[10] described a fatal case of anaemia which he suggested could be related to a disorder of the digestive system. In 1855 Addison[11] gave a detailed description of a patient with severe pernicious anaemia, and over the next fifty years a number of observations were made which related the occurrence of this particular form of anaemia to abnormalities of the digestive system, particularly the secretory glands in the stomach. The name 'progressive pernicious anaemia' was first used by Biermer[12] in 1872, but the cases he described may not all have been true pernicious anaemia.

The cause of the disease was not traced to a factor in the diet until the twentieth century. Possibly, the thinking of the late nineteenth century about disease was dominated by the discoveries of Pasteur and Koch on the infectious, or bacterial origins of illness, and so the earlier suggestions that anaemia was caused by defective diet or digestive disorders were neglected[9]. At the start of the twentieth century, the concept of nutritional deficiency was recognised, with work on beri-beri in Java, and experiments using purified diets in animals. Trace amounts of dietary accessories were shown to be required, and these were named 'vitamins' in 1913.

Studies by Whipple[13], on chronically bled dogs, suggested that red meat, especially liver, was effective in enhancing haemoglobin function. This effect is now known to be because of the high iron content of these foods, but before this was established, diets rich in liver were given to patients suffering from various types of anaemia. Pernicious anaemia sufferers, however, did not show consistent remission of the disease when a moderate amount of liver

was included in their diets. It was not until 1926 that Minot and Murphy[14] reported a consistent gain in the red blood cell count of patients who had eaten at least 120 to 240 g of liver daily. Whipple, Minot and Murphy received the Nobel Prize for Medicine for this work in 1934.

The search for the 'anti-pernicious-anaemia factor' was begun in 1927. Cohn and co-workers[15] undertook the fractionation of liver, and Minot tested the fractions on patients. In 1928 a liver fraction in the form of a yellow powder was prepared, of which 13 g contained the activity of 300 g of liver. A dose of this each day was much more palatable than large amounts of raw or lightly cooked liver.

Simultaneously, Castle[22] investigated the digestive disorders associated with pernicious anaemia. He showed, in 1929, that patients exhibited a much better response to liver or meat if normal human gastric juice was added to their diets. He therefore suggested that pernicious anaemia sufferers fail to secrete a substance, which he named 'intrinsic factor', which aids the uptake of 'extrinsic factor', later shown to be vitamin B_{12}. Intrinsic factor was later found to be a protein which binds to cobalamins in the intestine and aids their absorption through the gut wall. This mechanism is presumably necessary because the small concentration of vitamin B_{12} in the diet, and the large size of the cobalamin molecule, make the absorption of the vitamin by simple diffusion too slow to be effective.

The isolation of vitamin B_{12} progressed over the fifteen years following the work of Cohn, Minot and Murphy, in several laboratories. It was aided by the simultaneous development of techniques for the fractionation of proteins, such as salting out with ammonium or

magnesium sulphate, and, especially, by the development of chromatography, but it was hindered by the lack of patients on which to test the various fractions, and by the absence of any other test of its activity. Eventually, a microbial assay was developed[16], but it was extremely qualitative, and was used only in the very late stages of the isolation by the group from Merck[18].

By 1936, a fraction was purified (by adsorption and desorption from charcoal) by Laland and Klem[17], which was effective when 0.7 mg was injected. In 1939, an even more purified fraction was sealed on a microscope slide. This work, however, was not continued because of World War 2, and was neglected by other workers. Many years later the slide was re-examined, and was found to contain crystals of vitamin B_{12}, unrecognised earlier.

The actual isolation of vitamin B_{12} as cyanocobalamin was achieved in 1947 by Folkers and associates[18] at the Merck Laboratories in America, from liver and from fermentation mixtures almost simultaneously. They were followed within a few weeks by Lester Smith and Parker[19] at the Glaxo Laboratories in England. Once the bright red crystalline substance had been isolated, later investigators could isolate the vitamin much more easily, by following the red fractions through the isolation procedures. Ten years later, Barker and co-workers isolated the active coenzyme, adenosylcobalamin[20, 1].

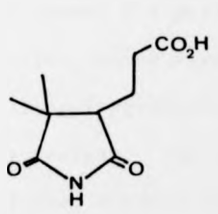
Although cyano- and hydroxocobalamin are isolated commercially from cultures of *Streptomyces griseus* and *S. aureofaciens* among others, as by-products of antibiotic production, the coenzymes are generally made by partial synthesis, adding the β-ligand artificially.

1.2.2 Structure elucidation

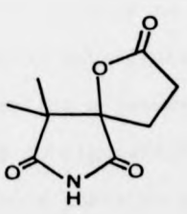
The determination of the structure of vitamin B_{12} was also made possible by the development of techniques never before used on such a large molecule, notably, X-ray crystallography. The ten years between the isolation of crystalline vitamin B_{12} and its final structure elucidation by Hodgkin using X-ray crystallography[23,24] was a time of intensive work in many laboratories, using the techniques of classical organic chemistry to study the degradation products of the vitamin.

The group at Merck[25] elucidated the structure of the 5,6-dimethylbenzimidazole group and its associated riboside, and the structure and configuration of the attached (*R*)-1-amino-2-propanol. It was established by a number of groups that B_{12} was not a peptide, as had at first been thought, and that it contained six primary amide groups, of which three were more labile than the others[26]. It was also shown that there is an amide linkage to the nucleotide side chain, but the identity of the chromophore surrounding the cobalt remained unclear.

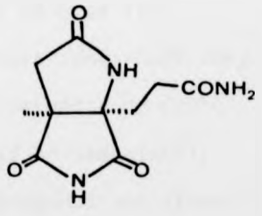
Eventually, controlled oxidation of the vitamin allowed the isolation[27] of the nitrogen-containing products (1) and (2), derived from ring C. Later, (3) was isolated[28], derived from ring B.



(1)



(2)

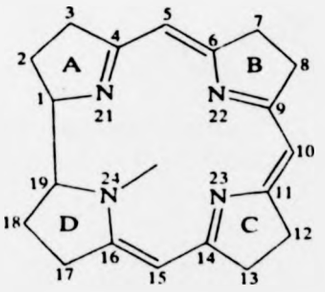


(3)

These were the first indications that pyrrol-like structures existed in the vitamin, but it was only when the X-ray structure was interpreted in 1954 that all the various pieces of evidence could finally be reconciled. It was a landmark in the history of X-ray crystallography, as never before had the structure of such a large molecule been determined by this technique, and it opened the way for the study of much larger molecules such as proteins, nucleic acids and viruses.

Crystals of cyanocobalamin, the selenocyanate and the hexacarboxylic acid were all used to solve the structure[29,30].

The molecule was found to contain an almost planar conjugated system, the corrin (4), resembling porphyrin, but more reduced, and with a direct link between rings A and D. In addition, the side



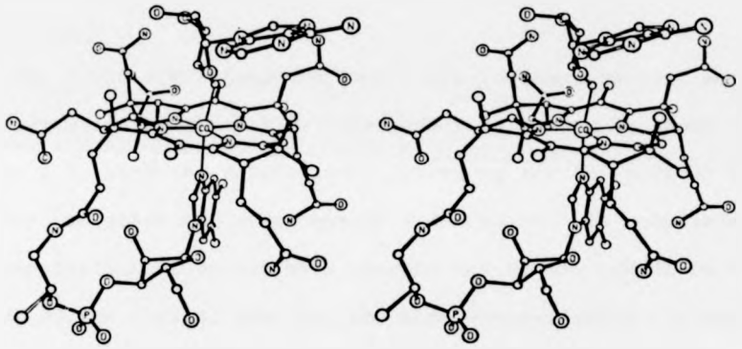
(4)

chains around the periphery are related to those of type III porphyrins, being asymmetrically substituted (short-long-short-long-short-long-long-short), suggesting a common biosynthetic or evolutionary origin. The absolute configuration could be immediately established because the molecule contains aminopropanol and ribose of known absolute configuration, and this was checked by X-ray studies

using the anomalous dispersion effect[23].

The structure of the coenzyme, 5'-deoxy-5'-adenosylcobalamin (= adenosylcobalamin) was also established by X-ray studies[32] before it was solved by chemical means. It was found to contain the previously unsuspected cobalt-carbon bond (2.03 Å)[33]. The three-dimensional structure is shown in Figure 1.2, and is further discussed in Chapter 5, on the crystallography of cobalamins.

Figure 1.2 from P. G. Lenhert, *Proc. Roy. Soc.*, **A303**, 45 (1968).



Stereoscopic view of the crystal of AdoCbl.

The cobalt atom is octahedrally coordinated by the corrin nitrogen atoms, the 5,6-dimethylbenzimidazole and the remaining ligand in the β-position. Its chemistry is dominated by the + 1, + 2, and + 3 oxidation states, and its ability to stabilise low spin complexes in any of these states. In alkylcobalamins, the cobalt atom is conventionally considered to be in the + 3 oxidation state, with negative charges residing on the phosphate, one of the corrin nitrogens and on the alkyl ligand. The same applies for

hydroxo- and cyanocobalamin. Aquocobalamin is the protonated form of hydroxocobalamin, and requires a counter-ion to balance the charge.

Reduction of cobalamin(III) with one electron leads to the 5-coordinate, paramagnetic, cobalamin(II), and further reduction gives cobalamin(I), which is one of the most nucleophilic species known. Reduction in acidic solution gives hydridocobalamin[34], which can be considered as protonated cobalamin(I), although the site of protonation is not agreed[34,35] (see Chapter 6).

1.3 Nomenclature

The IUPAC-IUB recommendations[36] are followed in this work and are summarised in Table 1.1. The numbering system is shown in figure 1.3, with the conventional lettering for the pyrrole rings and the acetamide and propionamide side chains. In cobalamins, the 5,6-dimethylbenzimidazole base coordinates to the cobalt on the α-side of the corrin, that is, the side towards which the methyl group on C1 points, while the remaining ligand occupies the β-position.

Figure 1.3

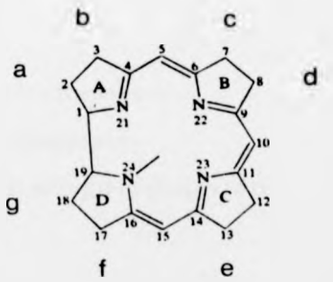
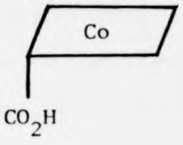
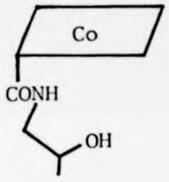
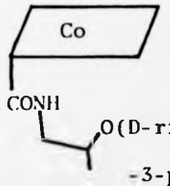
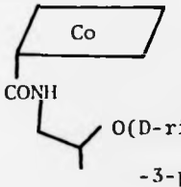


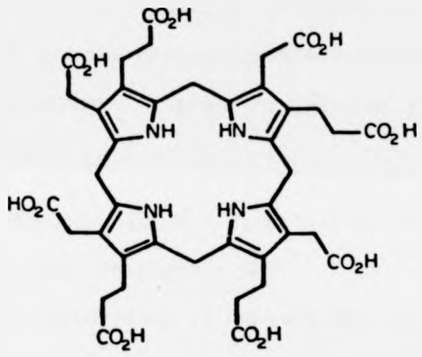
Table 1.1 IUPAC-IUB recommended nomenclature

	Structure	Name	
1.	Skeleton	Corrin	
		Heptaacid	Heptaacid hexaamide
2.		Cobyrinic acid	Cobyric acid
3.		Cobinic acid	Cobinamide
4.		Cobamic acid	Cobamide
5.		-	Cobalamin
6.	Cobalamins in which a further organic group is covalently ligated to cobalt		X-ylcobalamin

1.4 Biosynthesis of cobalamin

1.4.1 The corrin macrocycle[37]

The order of the side-chains around the corrin suggests a common biosynthetic origin of corrin with porphyrins and chlorins, and this was shown to be true by the incorporation of intact uroporphyrinogen-III (urogen-III) (5) into cobyrinic acid, in cell-free preparations of *P. shermanii*[38]. Uro'gen-III is known to be a precursor of porphyrin and chlorin, and is ultimately derived



(5)

from glycine and succinyl-CoA, *via* aminolevulinic acid and porphobilinogen. The use of ^{13}C-labelled precursors, and the advent of ^{13}C NMR spectroscopy allowed the elucidation of the pathway by which uro'gen-III becomes cobyrinic acid. The direct link between rings A and D is made by the methylation of C20 of uro'gen-III, followed by the extrusion of acetic acid. All the methyl groups in cobyrinic acid, except for that on C12 α, are derived from methionine[37] (the usual biological methylating agent), and all the other carbon atoms in the macrocycle are derived from aminolevulinic acid, including the α-methyl group on C12.

Detailed studies using labelled precursors and isolation of intermediates by Battersby and co-workers[37] are in progress to follow the order and stereochemical course of the methylations, and the introduction of cobalt. This last occurs very late in the biosynthetic pathway, and the conversion of the heptaacid to the heptaamide occurs after the insertion of cobalt.

1.4.2 The nucleotide loop[39]

The aminopropanol group appears to be derived from L-threonine by decarboxylation[39]. The resulting cobinamide is activated[40] by the addition of GDP and the dimethylbenzimidazole nucleotide is added to give cobalamin phosphate[41] (phosphorylated at R5 of the nucleotide) (Equation 1.1).

Equation 1.1 GDP-cobinamide + dimethylbenzimidazole nucleotide → cobalamin phosphate + GMP

The dephosphorylation step to convert the cobalamin phosphate into cobalamin has also been demonstrated in bacterial systems[42].

1.4.3 The β-ligand[39]

The adenosyl ligand is probably added soon after the corrin macrocycle is completed. Adenosylated intermediates such as adenosylcobyric acid and adenosylcobinamide have been isolated[43], although a wide range of corrin substrates, including cobalamins, can be adenosylated. Enzymes that catalyse the adenosylation of cobalamins are present[39,44] in the mitochondria of eukaryotic cells, although these lack the ability to make the corrin macrocycle or the nucleotide loop. Presumably, eukaryotes have to be able to repair the degraded coenzyme, while they have lost the ability to synthesise

the whole molecule, because of the lability of the cobalt-carbon bond. Methylcobalamin is produced in eukaryotic cells as a product of the methionine synthetase reaction, and possibly by other routes as well[39].

Attachment of the adenosyl group to cobalt requires the reduction of cobalamin(III) to cobalamin(I). This has been shown to proceed *via* cobalt(II) and to require two separate flavoproteins, one for each single-electron reduction, with NADH as the electron donor in both cases. Both enzymes require FAD (or FMN)[39].

The alkylating agent is ATP, which is an unusual role for this molecule, although it also acts in this way in the alkylation of methionine to give *S*-adenosylmethionine. The purified alkylating enzyme from *C. tetranomorphum* is not specific for ATP, but uses CTP and other nucleoside triphosphates as well, so clearly some controls exist in the cell to ensure that only the adenosyl group is attached to corrinoids[46,39].

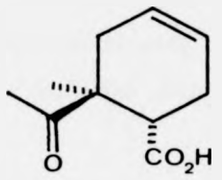
1.5 Total Synthesis

Once the structure of the vitamin had been determined, attempts were begun to synthesise it. It had already been established[47] that the groups attached to the propionamide side-chain of the D-ring, to make the nucleotide loop, can be attached to the free carboxyl group of cobyric acid, and so a synthesis of this compound would formally constitute a synthesis of the vitamin. Cobyric acid contains nine chiral centres, and the novel macrocyclic corrin, which had not been synthesised before.

Work was started between 1950 and 1960 in a number of laboratories, but the final successful syntheses were achieved by Woodward and associates at Harvard, and Eschenmoser and associates in Zurich[48].

One strategy involved the collaboration of both groups; the A-D half of the molecule was made in Cambridge and the B-C half in Zurich, and these were coupled to give cobyric acid (with the carboxyl group on the f-side chain protected as a nitrile).

The Zurich group also devised a synthesis by a photochemical pathway, using both enantiomers of the cyclohexene derivative (6) as the chiral precursors of all four pyrrole rings. This synthesis



(6)

illustrated the Woodward-Hoffman rules of orbital symmetry, because photochemical cyclisation, aided by certain metals, led to the desired *trans* A-D ring junction[49]. The rules had earlier been discovered during the synthesis of the A-D portion of B_{12} at Harvard.

By the end of 1972, cobyrinic acid had been synthesised and fully characterised, but the preparation of totally synthetic vitamin B_{12} was continued until, in 1973, crystals of pure cyanocobalamin were obtained[50]. These were identical in all respects to the natural vitamin, and were also totally biologically active.

The synthesis of vitamin B_{12} was a landmark in the history of synthetic organic chemistry. It not only showed that the proposed structure of the molecule was correct, but it also allowed the synthesis of a wide range of labelled analogues for use in biosynthetic studies. Furthermore, it paved the way towards the laboratory

synthesis of natural products containing many chiral centres, which is commonplace today.

Such a long and complicated synthesis could not be used to make the vitamin commercially. The most economical way of obtaining it is still by isolation from fermentation mixtures.

1.6 Vitamin B_{12} model compounds

A discussion of the mechanism of action of vitamin-B_{12}-dependent enzymic reactions must include some assessment of the compounds used as models of cobalamins, since much of the evidence for the various possible mechanisms has been obtained using non-cobalamin analogues of vitamin B_{12}.

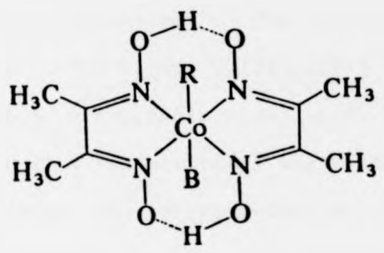
There are several reasons for the extensive use of model compounds in studies of vitamin B_{12}. Firstly, there was a lack of knowledge about the properties of organocobalt compounds, and so it was important to find out how general the observations were. Secondly, certain investigations cannot be carried out using the natural coenzyme, or its derivatives (for instance, the quite stringent steric requirements limit the size of the ligands that can be attached to cobalt). Thirdly, until recently, alkylcobalamins could not be well characterised spectroscopically; and fourthly, the expense of natural vitamin B_{12} precludes its use on a large scale.

The model compounds used are, in general, cobalt complexes. They share with vitamin B_{12} the ability to exist as stable, low spin, Co(I), Co(II) and Co(III) complexes, and to form alkylcobalt complexes containing a cobalt-carbon σ-bond.

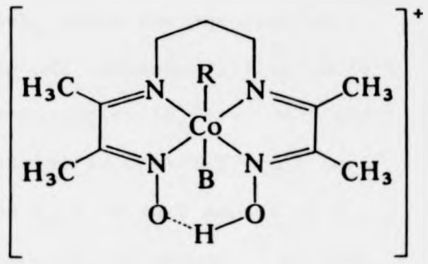
Some of the most commonly used model complexes are shown in Figure 1.4. The cobaloximes[21] have been most extensively used as model systems, but certain workers[51] suggest that the Costa

complex (figure 1.4) is closer in its electrochemical and other properties to cobalamins, and is therefore to be preferred.

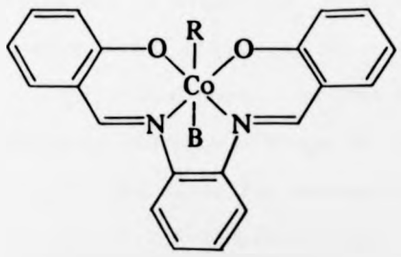
Figure 1.4



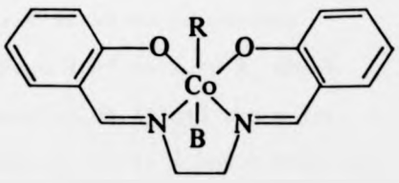
(A) Cobaloxime



(B) Costa complex



(C) [R—Co(SALOPH)B]



(D) [R–Co(SALEN)B]

Comparative studies[51,52] on the various complexes have shown that while they mimic vitamin B_{12} in certain respects there are quantitative differences. In general, cobaloximes and Schiff base compounds (e.g. Costa complexes) are the closest non-corrin analogues of cobalamins over a range of properties, but there are important differences.

Cobaloximes and Costa complexes form more stable organocobalt complexes than do cobalamins, and also they cannot mimic the steric interactions between the alkyl ligand and the corrin. In electrochemical properties, Costa complexes are closer to cobalamins than are cobaloximes[51]. The Co(III)/Co(II)$E_{\frac{1}{2}}$ value for aquocobalamin is -0.042 V, and Co(II)/Co(I) for base-off cobalamin(II) is -0.74 V. These are closely modelled by the Costa complex ($E_{\frac{1}{2}}$ = -0.04 V and -0.71 V respectively) while the cobaloxime is more difficult to reduce, the corresponding values being $E_{\frac{1}{2}}$ = -0.40 V and -1.12 V respectively. The differences are taken by the authors[51] to show that the equatorial ligand in the Costa complex interacts with the cobalt atom in a similar way to that of the coenzyme. The Costa complex has the same formal charge on its equatorial ligand as the corrin has in vitamin B_{12} (-1) while the cobaloxime has a corresponding formal charge of -2.

The Co-C bond lengths have been measured in various complexes and show a range of values from 1.93 to 2.20 Å, which includes the value for adenosylcobalamin (2.05 Å) and those for (*R*) and (*S*)-2,3-dihydroxypropylcobalamin (2.00(2) Å and 2.08(3) Å, respectively) which are presented in this work (Chapter 5).

The cobalt-carbon bond dissociation energy has apparently recently been measured[53] (120 kJ mol^{-1}). Previously, all the data had been obtained with model compounds[54,55], in particular cobaloximes and Schiff base complexes, which have bond dissociation energies in the range 80 - 130 kJ mol^{-1}, that is, quite low compared with other transition-metal-carbon σ-bonds (∿ 200 kJ mol^{-1}).

In view of the differences, it is important to test the conclusions derived from model studies on the relevant B_{12} systems themselves. The present work seeks to extend the studies of

Golding *et al.*[31,56] on cobaloximes, to cobalamins, and to improve the characterisation of alkylcobalamins in order to do this.

1.7 Biological role of vitamin B_{12}

1.7.1 Enzymic reactions

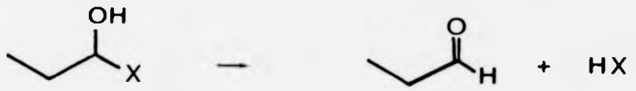
Two main types of vitamin-B_{12}-dependent reactions occur. One type involves methyl transfer of various kinds, and uses methylcobalamin as an intermediate methyl carrier[57]. Such transfers are involved in the biomethylation of metals and the biosynthesis of acetate and methionine. The only known mammalian enzyme requiring methylcobalamin is 5-methyl-H_4-folate homocysteine methyltransferase, which is involved in methionine metabolism. These systems have been extensively studied[57] but are not discussed further here.

The other type of reaction involves the rearrangement shown schematically in Equation 1.2 in which a hydrogen atom and a group, X,

Equation 1.2

on adjacent carbons, are exchanged. Group X can be an electron-withdrawing group such as hydroxyl or amino, in which case a second reaction involving the loss of water or ammonia can take place (Equation 1.3) to give an aldehyde.

Equation 1.3



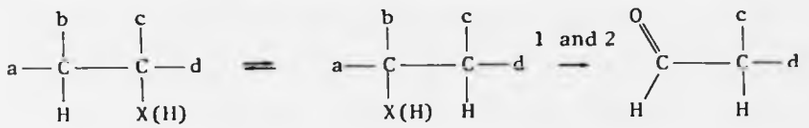
The enzymic interconversions are shown in Table 1.2. Adenosylcobalamin-dependent ribonucleotide reductase is also thought to be related to these reactions.

The mechanisms of action of all these enzymes are believed to have features in common. Since the present work is concerned with diol dehydratase, this enzyme is considered in detail, and the mechanistic findings relevant to it and to other B_{12}-dependent enzymes are described below.

1.7.2 Propanediol dehydratase (EC 4.2.1.28)[58]

This enzyme is produced by several strains of bacteria, and was first isolated by Abeles *et al.*[59] from *Aerobacter aerogenes*. They found that cells grown anaerobically on glycerol could convert glycerol into β-hydroxypropanal, also 1,2-propanediol into propanal and 1,2-ethanediol into acetaldehyde. In intact cells, glycerol and propanediol react at about the same rate, but in cell-free systems, or with the purified enzyme, glycerol reacts at only 5 - 10 % the rate of propanediol. This is thought to be because although glycerol is a good substrate, it also rapidly inactivates the enzyme. A closely related enzyme, glycerol dehydratase (EC 4.2.1.30) has also been studied[60], and shown by immunochemical techniques to be different from propanediol dehydratase (hereafter known as diol dehydratase). Diol dehydratase[59] has a molecular weight of about

Table 1.2 Adenosylcobalamin-dependent rearrangements



	Enzyme	a	b	c	d	Migrating group X(H)
1(a)	Diol dehydratase	OH	H	H	Me or H	OH
(b)	Glycerol dehydratase	OH	H	H	CH_2OH	OH
2.	Ethanolamine ammonia lyase	OH	H	H	Me or H	NH_2
3.	(*R*)-Methylmalonyl-CoA mutase	Me or H	H	CO_2H	H	COSCoA
4.	(*S*)-Glutamate mutase	H	H	CO_2H	H	$CHNH_3^+CO_2^-$
5.	α-Methyleneglutarate mutase	H	H	CO_2H	H	$(C=CH_2)CO_2^-$
6.	Aminomutases e.g. leucine amino mutase	Me_2CH	H	CO_2H	H	NH_2
7.	Ribonucleotide reductase (ribonucleotide → deoxyribonucleotide)					

230,000, and can be separated into two components, F and S, with molecular weights 26,000 and 200,000 respectively. Neither component can catalyse the dehydration of 1,2-diols on its own, or bind firmly to either adenosyl- or cyanocobalamin. The S component can be dissociated into four subunits, each with a different molecular mass, which suggests that it is composed of four different polypeptide chains. The enzyme requires a univalent cation, such as K^+, NH_4^+, Tl^+ or Rb^+, all of which have very similar ionic radii. Ions of larger or smaller ionic radius cause greatly decreased activity[61].

If the coenzyme is modified, the enzymic activity usually decreases. Toraya, Abeles *et al.*[62] showed that the propionamide residues on the periphery of the cobalamin are involved in the binding of the coenzyme to the protein, and also for transmitting the interactions between the protein and the cobalamin which destabilise the cobalt-carbon bond, which is important in the catalytic cycle. It has also been shown[63] that an analogue of adenosylcobalamin that contains a carbocyclic analogue of the ribose portion of the adenosyl ligand, is as active as the natural coenzyme. Thus, the ring oxygen of adenosyl is not involved in the stabilisation of any intermediates.

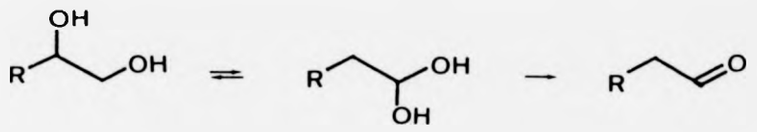
No vitamin-B_{12}-dependent enzyme has been crystallised or had its amino-acid sequence determined, so little is known about the structure or properties of the active site, although most, if not all, of these enzymes contain a thiol in the active site[64].

1.7.3 Substrate specificity and stereochemistry

The reaction catalysed by propanediol dehydratase involves the rearrangement of a 1,2-diol to a 1,1-diol, followed by enzymic

dehydration to give an aldehyde (Equation 1.4). This reaction is related to that of ethanolamine ammonia lyase, in which an amino group migrates, and ammonia is lost, to give an aldehyde.

Equation 1.4

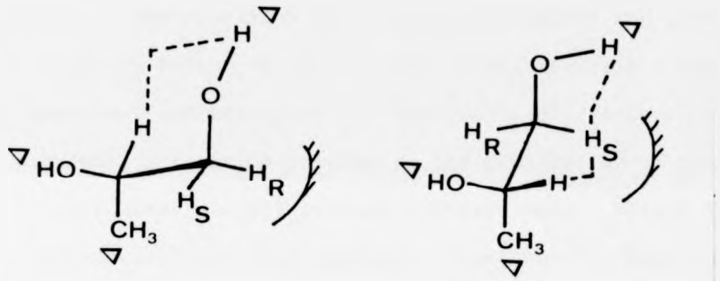


Propanediol dehydratase accepts various 1,2-diols as substrates, with (*R*)- and (*S*)-propane-1,2-diol and glycerol having the highest initial rates. Butane-2,3-diol is the only compound not containing a 1,2-diol which shows substrate activity[65]. The *meso*-isomer is converted into butanone, while the (2*S*, 3*S*)- and (2*R*, 3*R*)-isomers are compeditive inhibitors. This fits in with the stereoselectivity observed with (*R*)- and (*S*)-propane-1,2-diol, that is, the pro-*R* hydrogen migrates in the case of the *R*-isomer, and the pro-*S* hydrogen in the case of the *S*-isomer[66,67]. The enzyme is unusual in that it accepts both enantiomers of the substrate, but it has been shown[58] that it distinguishes between them (see also section 3.3.1) the *R*-isomer reacting at 1.7 - 1.8 times the rate of the *S*-isomer when the two isomers were presented separately, while the *S*-isomer reacts faster in compeditive reactions. This is because the *S*-isomer has a higher specificity constant (k_{cat}/K_m).

Abeles *et al.*[66,67] showed that the stereochemistry of hydrogen transfer depends on which isomer is reacting, as mentioned above, and that the hydrogen replaces the migrating hydroxyl group at C2 with inversion.

This stereochemistry can be explained by assuming that the diol binds to the enzyme at three points. Figure 1.5 shows how the pro-*S* or the pro-*R* hydrogen could be presented to the C5' position of the coenzyme, depending on the configuration at C2 of the substrate.

Figure 1.5



R *S*

▽ = protein binding site

The studies of Moore and Richards[65] on butanediols, which have methyl groups in the positions occupied by the hydrogens abstracted in the propanediols, provide further support for this type of binding.

The role of the adenosyl group as an intermediate hydrogen carrier was also demonstrated by Abeles' group[68] who showed that incubation of the enzyme with a mixture of unlabelled ethane-1,2-diol and [1,1-$^{3}H_{2}$]propane-1,2-diol produced tritiated ethanal, showing

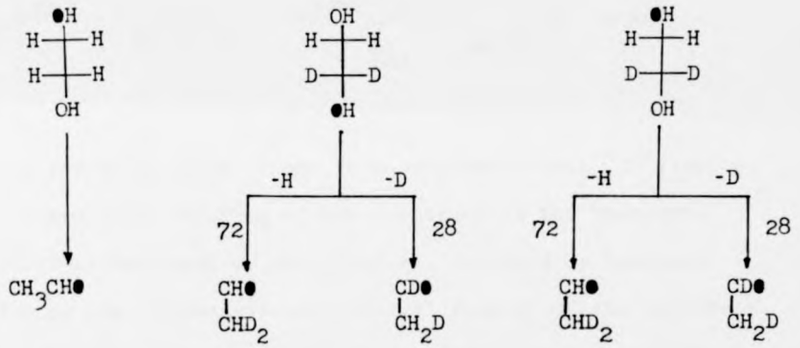
that intermolecular hydrogen transfer occurs. In addition, both 5'-hydrogens of the coenzyme were found to be labelled, and transfer of tritium from either of these positions to C2 of the product could occur. This demonstrated that at some point in the reaction, the two C5' protons and the proton from C2 of the product, all became equivalent. This could be explained by the intermediate formation of 5'-deoxyadenosine, by hydrogen transfer to C5' to give a methyl group. This was first demonstrated by Wagner *et al.*[69], in the reaction of diol dehydratase with glycolaldehyde, and then, by others, in the reactions with chloracetaldehyde and glycerol[58]. It was shown by Babior *et al.*[70] to be formed reversibly when ethanolamine ammonia lyase catalysed the conversion of 2-amino-1-propanol into propanal, a reaction related to the dehydration of propanediol.

A substantial overall primary kinetic isotope effect has been found for adenosylcobalamin-dependent reactions[71]. For diol dehydratase operating on propane-1,2-diol and [1,1-$^2H_2$]propane-1,-2-diol, the overall $k_H/k_D = 12$. This value is made up of the isotope effect for the abstraction of hydrogen from the substrate ($k_H/k_D = 10.8$), and the isotope effect for the abstraction of hydrogen from the methyl group of deoxyadenosine ($k_H/k_D = 2.2$).

The stereochemistry of the hydroxyl group migration was studied by Arigoni, Rétey *et al.*[72]. Using (*R*)- and (*S*)-[1-^{18}O]propane-1,-2-diol they obtained [^{18}O]propanal from the *S*-isomer, and unlabelled propanal from the *R*-isomer. This observation confirms the intermediacy of the 1,1-diol, and shows that not only is the initial transfer of the hydroxyl group stereospecific (or at least highly stereoselective), but also the dehydration of the intermediate 1,1-diol is stereospecific and therefore enzyme-mediated.

An important study by Arigoni and co-workers[73] using labelled ethane-1,2-diols showed that with this substrate, racemisation at both C1 and C2 takes place, in contrast to the findings with propane-1,2-diol. The use of ^{18}O-labelled substrates showed a random (statistical) retention of ^{18}O, and the use of dideuterated, ^{18}O-labelled, ethanediols provided evidence demanding the formation of a *gem*-diol before its dehydration to ethanol (Equation 1.5).

Equation 1.5



● = ^{18}O

49 % control

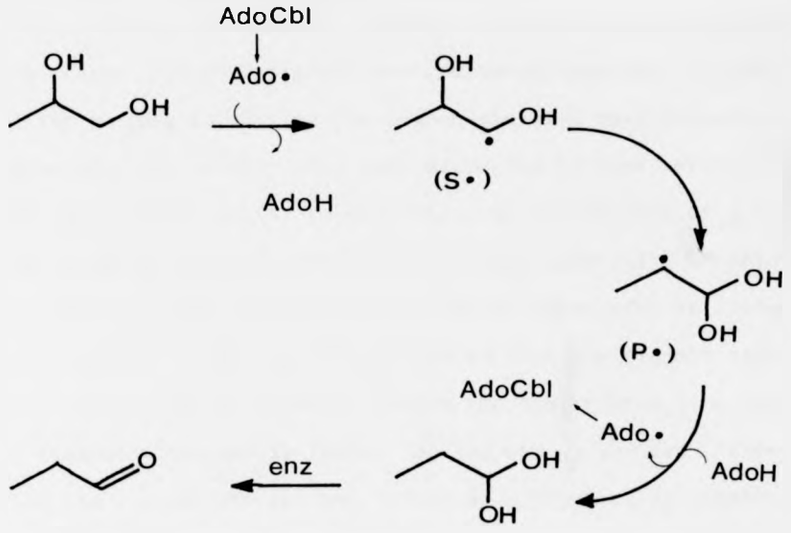
52 %

51 %

1.7.4 Mechanism of action

A mechanism that combines the various stereochemical findings is shown in Scheme 1.1 and a similar scheme can be written for the other B_{12}-dependent rearrangements.

Scheme 1.1: The diol dehydratase reaction



The evidence for this scheme is summarised below. In essence, the first event after binding of the substrate is the homolysis of the cobalt-carbon bond of the coenzyme, followed by hydrogen abstraction by the 5'-deoxyadenosylradical from C1 of the substrate. The substrate-derived radical (S•) then in some way becomes the product-like radical (P•), which takes back a hydrogen atom from the methyl group of 5'-deoxyadenosine to give the product, a *gem*-diol. This is further enzymically dehydrated to give propanal.

Some workers[74,75] do not agree with this scheme, arguing the intermediacy of radical species has not been proven, but the mechanisms that they suggest, also lack experimental evidence.

The initial homolysis of the cobalt-carbon bond was shown to occur by ESR studies[76,77], in which preparations of diol dehydratase in the presence of propane-1,2-diol were frozen, and gave signals characteristic of cobalamin(II) and an organic radical. Similar

observations were made with ethanolamine ammonia lyase[78] and ribonucleotide reductase[79] in the presence of their respective substrates. No such signals were observed, however, in the case of the enzymes catalysing the carbon-skeleton rearrangements[80]. Although these studies have been dismissed by some workers[74,75], the observations appear to be valid, as the doublet at g = 2.0 (the presumed organic radical) is not seen after the substrate has been consumed, and is altered if the substrated is isotopically substituted. Boas *et al.*[81] calculated that the organic radical is at a distance of at least 10 Å from the cobalt atom, in the case of ethanolamine ammonia lyase. Abeles and co-workers[76] showed that the radical species are formed at a kinetically competent rate in the conversion of propane-1,2-diol into propanal.

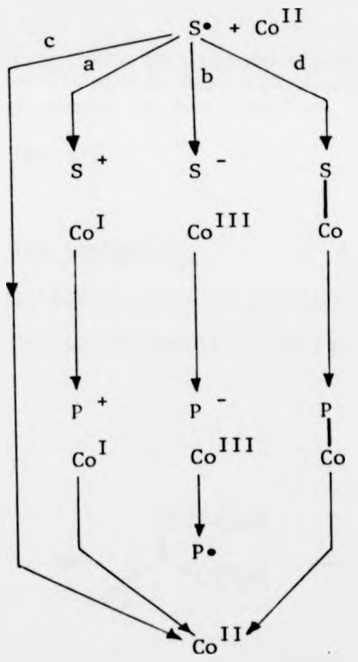
It is likely that the binding of substrate to the enzyme causes the labilisation of the cobalt-carbon bond by a conformational change in the protein. The cobalamin is already under considerable steric strain, with a Co-C-C bond angle of 125 ° (compared with 109 ° for a normal tetrahedral angle)[33]. This large angle is thought to be due to the steric interaction of the adenosyl group with the peripheral groups on the corrin, although it has been suggested[82] that it is evidence of three-centre 'agostic'[202] bonding involving the cobalt, C5' and H5' atoms.

In the absence of substrate, very little of the Co-C bond of the coenzyme is dissociated, but up to 60 % dissociates once the substrate binds. The adenosyl radical is presumably prevented from cyclisation and from other reactions by tight binding to the enzyme. Alternatively, transfer of hydrogen from the substrate to C5' of adenosine may be concerted with the cleavage of the cobalt-carbon bond[83].

1.7.5 The rearrangement of S• to P•

This step is the least well understood of any in Scheme 1.1, and is the focus of much study. The possible pathways for this step are shown in Scheme 1.2, and include the direct rearrangement of the radicals, electron transfer to give either a carbocation ion or a carbanion, and the formation of organocobalt species. Since the migrating groups in the different enzymic rearrangements vary widely, the mechanism of this step may be different in each case.

Scheme 1.2: Possible pathways for S• → P•

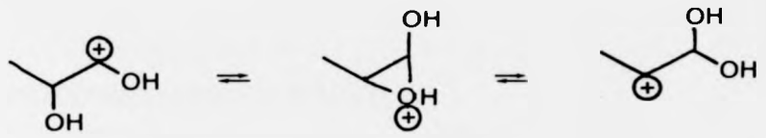


The following sections summarise the evidence for each of the possible pathways, with particular reference to the mechanism of diol dehydratase.

1.7.5.a Rearrangement involving carbocations

Pathway a has been used to explain the action of diol dehydratase[71] and ethanolamine ammonia lyase[84] with mechanisms such as that in equation 1.6.

Equation 1.6

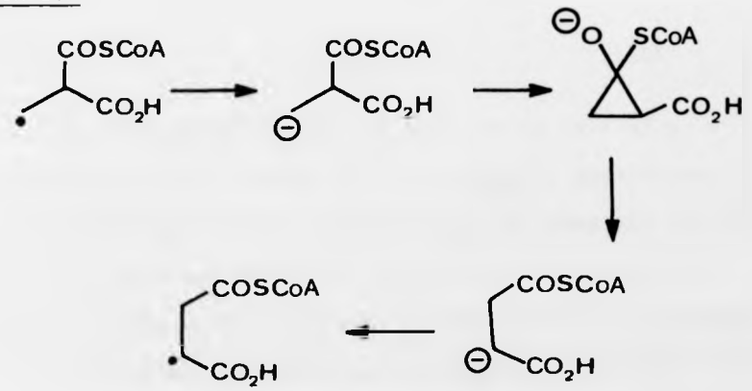


Analogous pathways could be used to explain the migration of the carbon-containing groups in for instance, methylmalonyl-CoA mutase or glutamate mutase.

1.7.5.b Rearrangement involving carbanions

This pathway has been invoked to explain the methylmalonyl-CoA mutase reaction *via* an intermediate cyclopropanol anion (Equation 1.7).

Equation 1.7



The model studies by Dowd[85], Rétey[86], Scott[87] and Schrauzer[88], suggest that the carbon skeleton-rearrangements may proceed *via* cobalt-stabilised anionic species, and may involve cobalt-carbon σ-bonded species. However, many of these studies could also be interpreted as evidence for either organocobalt species, radical intermediates or anionic species[52,89] so firm conclusions cannot be drawn.

1.7.5.c Rearrangement involving radicals

[1,2]-Shifts of unstabilised radicals are not favourable (Equation 1.8), the bridged species (II) being much higher in energy than the acyclic isomers (I) and (III). If the radicals (I) and (III) interconvert, they do so by a dissociation-recombination pathway[89].

Equation 1.8

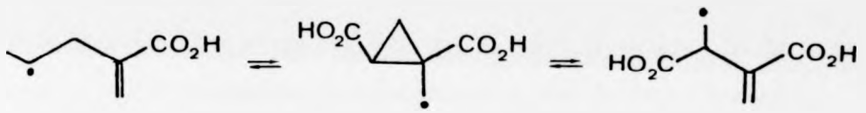
(I) (II) (III)

If X is a third-row element, however, or is part of a conjugated system, the radical (II) is relatively more stable, and intramolecular [1,2]-shifts can take place, but they are not found in the case of unsubstituted first-row elements. Therefore an unaided rearrangement of a substrate-derived radical or carbanion can be discounted for diol dehydratase, ethanolamine ammonia lyase,

amino mutases and glutamate mutase.

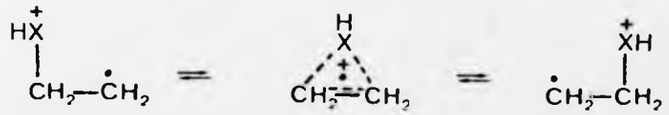
For α-methyleneglutarate mutase, radical intermediates alone may be present, since but-3-enyl and cyclopropylmethyl radicals are known[90] to interconvert at a rate compatible with that of the enzymic reaction. Equation 1.9 shows a possible pathway, which is supported by the model studies of Chemaly and Pratt[91], Golding and Mwesigye-Kibende[92], and Johnson and co-workers[107], using substituted butenyl- and cyclopropylmethylcobaloximes, but these studies have also been interpreted in terms of Co-C bonded intermediates[107].

Equation 1.9



Ab initio molecular orbital calculations by Golding and Radom[93] showed that protonation of the migrating group X (X = OH, $-CH_2R$, NH_2), could lower the energy of the intermediate bridged species to a point where the rearrangement becomes feasible (Equation 1.10).

Equation 1.10



For X = OH, the bridged (protonated) species is 34 kJ mol^{-1} less stable than the open species, whereas for X = NH_2, it is 139 kJ mol^{-1} less stable. For X = CH_2, the bridged species (the radical cation of cyclopropane) is 50 kJ mol^{-1} more stable than the open species. If the species with X = OH is substituted with hydroxyl or methyl on one carbon atom, the bridged species becomes energetically indistinguishable from the open chain species. These findings are relevant to the mechanisms of diol dehydratase, ethanolamine ammonia lyase, and methylmalonyl-CoA mutase, since they suggest that these migrations can occur without participation from the cobalt, so long as protonation of the substrate radical can occur.

An alternative radical reaction that has been suggested[53], is the deprotonation of the $HO\dot{C}H\text{-}CH(OH)CH_3$ radical to give $^{-}O\text{-}\dot{C}H\text{-}CH(OH)CH_3$ anion, which decomposes spontaneously to $CHO\text{-}\dot{C}H\text{-}CH_3$. Ethane-1,-2-diol radical has a pKa of about 10 while the parent alcohol has a pKa of about 15. The radical undergoes very smooth acid- or base-catalysed dehydration to give ethanal (Equations 1.11 and 1.12).

Equation 1.11

$$HOCH_2\dot{C}HOH \overset{H^+}{\rightleftharpoons} H_2{}^{+}O\text{—}CH_2\dot{C}HOH \rightarrow H_2O + H^+ + \dot{C}H_2CHO \xrightarrow{+\,H\bullet} CH_3\text{—}CHO$$

Equation 1.12

$$HOCH_2\dot{C}HOH \overset{OH^-}{\rightleftharpoons} HOCH_2\dot{C}HO^- \longrightarrow \dot{C}H_2CHO + HO^- \xrightarrow{+\,H\bullet} CH_3CHO$$

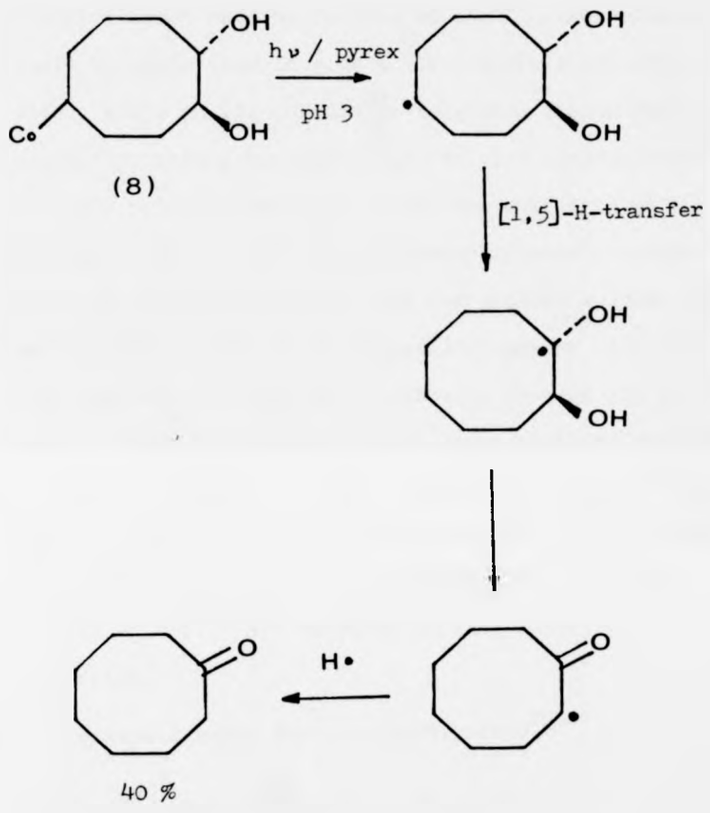
Hydroxyl radicals can abstract a hydrogen atom from C1, C2 or C3 of propane-1,2-diol[95]. Under acidic or basic conditions, the radical resulting from H-abstraction from C1 is converted into

propanal, by a similar pathway to Equation 1.11 and 1.12. Amino-alcohols react in a similar way[96], with the 2-amino-1-hydroxyalkyl radicals decomposing to ammonia and the 1-formylalkyl radical even at pH 7. Equations 1.11 and 1.12, when applied to the diol dehydratase reaction, do not explain the labelling results of Arigoni and co-workers[72], which require the formation of an intermediate *gem*-diol. Proponents of this theory[53] have to assume that water adds back to the formylalkyl radical, followed by a further dehydration.

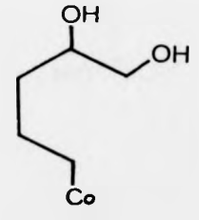
Model studies to ascertain whether an alkyl radical can convert a 1,2-diol into an aldehyde started with the observations that anaerobic photolysis of alkylcobalt species including cobalamins in the presence of ethane-1,2-diol, led to the production of ethanal[97,98,99]. Models designed to mimic the stereospecific abstraction of hydrogen from C1 of a 1,2-diol were synthesised by Golding *et al.*[31,56]. These are described in more detail in Chapter 6, but in summary, photolysis of the dihydroxyalkylcobaloximes (*e.g.* (7) and (8)), under acidic conditions, led to the production of pentanal from (7), and cyclooctanone from (8). The proposed pathway for (8) is shown in Scheme 1.3, and includes a [1,5]-hydrogen transfer. [1,6]-H shifts were also possible, but less favourable, as shown by the 4 : 1 mixture of hexan-2-one and hexanal produced by the photolysis of 5,6-dihydroxyhexylcobaloxime.

Johnson *et al.*[99] showed that the conversion of ethane-1,2-diol into ethanal could also take place at pH 7.4 and 11.2 as well as at pH 2.0. Müller and Rétey[100] demonstrated the catalytic conversion of the diastereomeric 6,7-dihydroxycycloundecyl iodides into cycloundecanone in the presence of sodium borohydride and a catalytic amount of a cobalt complex, such as cobaloxime, cobalamin,

Scheme 1.3 Formation of cyclooctanone[56]



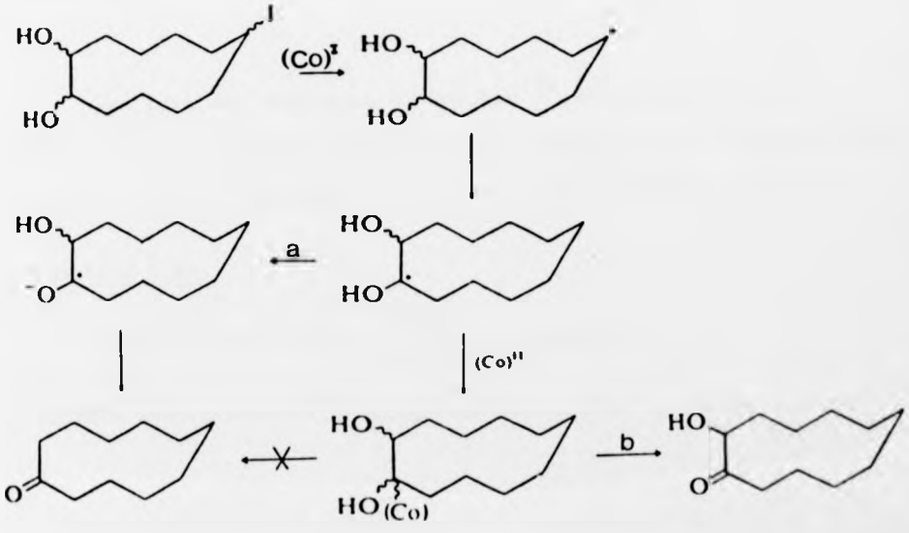
(8)



(7)

Costa complex, and even cobalt(II) chloride. Cycloundecanone was formed in up to 75 % yield, which corresponded to 75 catalytic cycles at the concentration used (1 % of the Costa complex). As in the cyclooctyl model used by Golding *et al.*[56], a transannular hydrogen shift is postulated to give a 1,2-dihydroxycycloalkyl radical, which, above pH 11, dehydrates to give a cycloundecanone radical, which abstracts a hydrogen atom, to give cycloundecanone. The yield of this product decreases as the concentration of the Costa complex increases. 2-Hydroxyundecanone is seen at higher concentrations of the Costa complex, and the authors suggest that it is formed by combination of the cobalt(II) species with the 1,2-dihydroxy radical, followed by elimination (Scheme 1.4 b). If this is the correct mechanism, then it seems that the formation of a 1,2-dihydroxyalkylcobalt species, postulated by some[101] as an intermediate in the diol dehydratase reaction, in fact prevents the desired reaction, rather than facilitating it. The model studies by Finke *et al.*[102] are relevant to this question, and are discussed below

Scheme 1.4 Catalytic model for diol dehydratase[100]

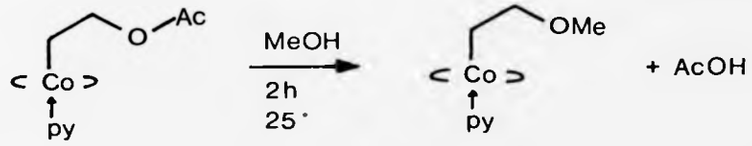


1.7.5.d Rearrangements involving organocobalt species

The stereoselectivity of the diol dehydratase reaction led various workers to propose that the radical S• formed a σ-bond with the cobalt(II) species to give an alkylcobalamin. The hydroxyl-group migration could be mediated by the cobalt atom.

Babior[103] first suggested that the diol dehydratase and ethanolamine ammonia lyase reactions proceed *via* a π-complex (9) since it was known[83] that 2-acetoxyalkyl(pyridine)cobaloximes rapidly undergo alcoholysis in neutral solution, probably *via* a π-complex (Equation 1.13).

Equation 1.13



The idea was developed by Dolphin[101] who demonstrated the formation of alkylcobalamins from hydroxocobalamin and nucleophilic olefins, again, presumably *via* a π-complex (Equation 1.14).

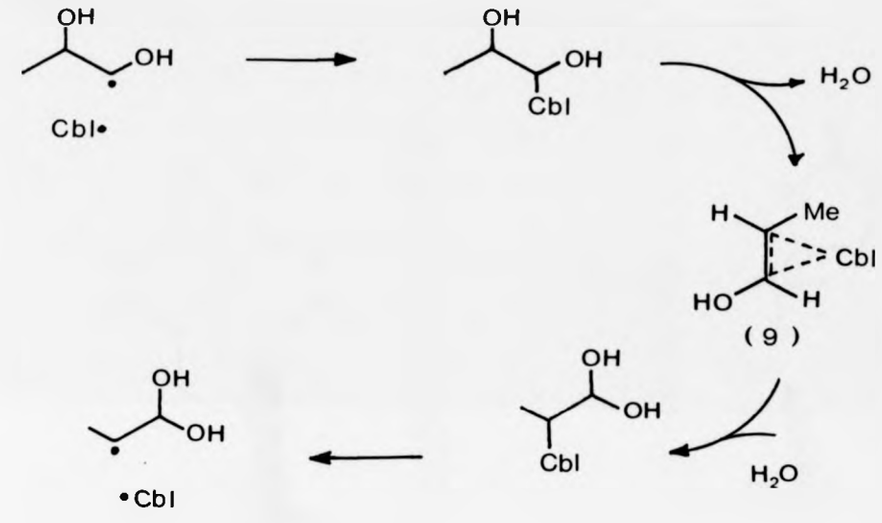
Equation 1.14

$$OHCbl + EtOCH{=\!=\!=} CH_2 \xrightarrow[Et_3N]{EtOH} (EtO)_2CHCH_2Cbl$$

Dolphin and co-workers[101] suggested the following scheme for the

diol dehydratase reaction (and a similar one for the ethanolamine ammonia lyase reaction) (Scheme 1. 5).

Scheme 1. 5

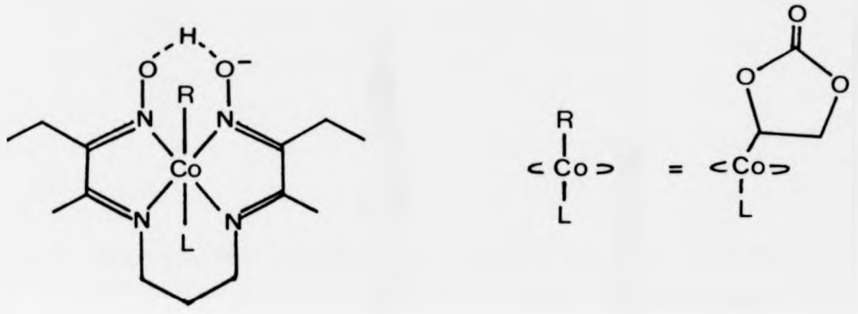


A number of studies have been designed to investigate whether alkyl-cobalt species are in fact intermediates in the enzymic reaction. Schrauzer[104] pointed out that the 2,2-dihydroxyethyl-cobalamin necessary in the reaction of ethane-1,2-diol by this mechanism, could dehydrate to formylmethylcobalamin (10). This

(10)

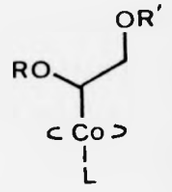
is too stable to be a catalytic intermediate, without considerable activation by the enzyme, and in any case, decomposes to give ethanal and hydroxocobalamin rather than the required cobalamin(II).

Finke *et al.* synthesised a protected 1,2-dihydroxyethyl-cobalt species (11) based on the Costa complex.[102]



(11)

Under basic conditions (pH 10 - 12), it was deprotected, and ethanal was produced in 95 % yield. The stoichiometry (particularly the observed 100 % yield of cobalt(II) complex) suggested the formation of a $\cdot CH_2CHO$ intermediate and the remaining hydrogen atom was found to come from the solvent (when it was benzyl alcohol, and probably when it was methanol). It did not seem to be produced by a π-complex mechanism leading to the formyl-methyl-cobalt species, since the authors used a π-complex route to prepare the formylmethyl-cobalt complex and found that it was too stable to participate in the ethanal-producing reaction. No formylmethyl-cobalt complex was found in the reaction mixtures. It was assumed (and experimental evidence is cited to support this) that the initial products of deprotection with MeO^- were (12a) and (12b).



a R = CO_2CH_3 R' = H

b R = H R' = CO_2CH_3

c R = H R' = H

(12)

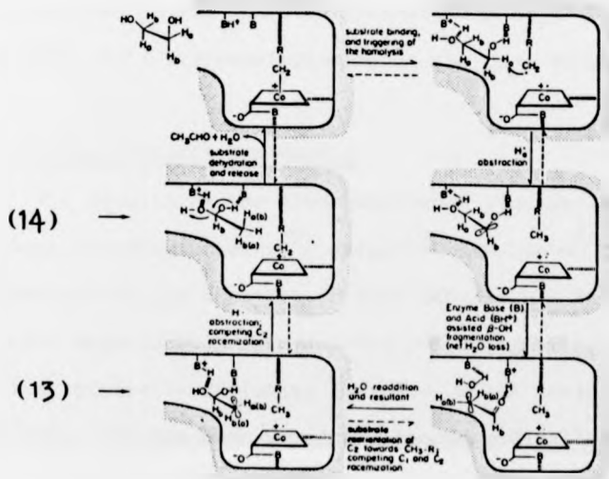
The other products were $CH_3OCO_2^-$ and $CH_3OCO_2CH_3$, each in about 50 % yield. Spin trapping experiments strongly suggested that the observed products came from freely diffusing radicals, that is, those produced from (12 a), (12 b) and (12 c) by homolysis of the Co-C bond, followed by escape from the solvent cage.

Addition of 1,5,6-trimethylbenzimidazole led to a change in the observed products. As more of the aromatic base was added, the ethanal yield decreased and the Co(I) yield increased (at the expense of Co(II)). The $CH_3OCO_2^-$ yield fell from 50 to 0 % while the $CH_3OCO_2CH_3$ yield rose from 50 to 100 %. By mass and charge balance a corresponding increase in hydroxyethanal was assumed. These results were interpreted to mean that base-on cobalt participation is not only unnecessary, it actually prevents the formation of ethanal, leading instead to a side reaction giving cobalt(I) and hydroxyethanal. This supports the studies of Mulac and Meyerstein[105], who showed that the pulse radiolysis of (12 c) led to cobalt(I) and hydroxyethanal.

In summary, it seems clear that even when the cobalt-carbon bond of a proposed intermediate (12 c) is already formed, a cobalt-assisted rearrangement to the desired product does not take place. Instead, all the results are consistent with the formation of the product (in this case, ethanal) only after homolysis of the cobalt-carbon bond, by a base-catalysed reaction.

Finke and co-workers[102,53] suggest that this is a good model for the diol dehydratase reaction, in which no organocobalt intermediates have been detected. They posulate a basic group in the active site of the enzyme, able to deprotonate the dihydroxyalkyl radical and catalyse the dehydration, to account for the discrepancy between the high pH values required for the model system (pH 10 - 12) and the optimum pH range for diol dehydratase (pH 6 - 10). The intermediate *gem*-diol required by the labelling studies has to be produced by the readdition of a molecule of water to the formylmethyl radical (13, Scheme 1.6), to give the product-like radical (14), which, after addition of H·, is dehydrated to propanal. The authors[53] suggest that this is not a non-essential step, as first

Scheme 1.6 Proposed pathway for diol dehydratase[53]



appears, but instead is used by the enzyme to reorientate the radical towards the coenzyme, so that it can repossess a hydrogen atom from the 5'-deoxyadenosine, and is prevented from indiscriminate radical abstractions within the protein. The two hydroxyl groups of the *gem*-diol are used as protein binding sites to the enzyme. The conversion of the carbonyl group into a diol may also serve to activate the radical by removal of any resonance stabilisation, thus facilitating the H-abstraction from the methyl group of 5'-deoxyadenosine.

The racemisation at C1 and C2 of ethane-1,2-diol[73] can also be explained by this mechanism[53]. The weakly-bound $\bullet CH_2CHO\ldots\ \overset{+}{H}B$- can racemise at the carbonyl carbon (C1) by presenting one or other face of the carbonyl for the readdition of H_2O. Presumably the extra methyl group in propane-1,2-diol provides some hindrance to this racemisation, although it is not clear why this should be.

The racemisation at C2 could occur either at this stage, or when the carbonyl has been hydrated. Again, the $\bullet CH_2$- group of the ethanediol intermediate has a lower barrier to rotation than does the CH_3-$\dot{C}H$- group of the propanediol intermediate, and this is reflected in the observed racemisation at C2 of ethanediol, and the > 88 % stereoselectivity in the case of propanediol.

1.7.6 The 'bound-radical' hypothesis

The results of the above-mentioned studies have led various authors (notably Golding[89], Arigoni[73] and Finke[53]) to suggest a mechanism for the reaction of diol dehydratase that does not involve organocobalt species, and yet does not require the intermediacy of freely diffusing radicals, which could cause havoc in the cell. It has been named the 'bound-radical' hypothesis by

Finke[53] and explains the observed stereochemical and kinetic data. Its proponents, however, are still not agreed on the precise mechanism of the rearrangement step, since model studies[102,56] support either an acid- or base- catalysed dehydration of a dihydroxyalkyl radical to give a formylalkyl radical. A dissociation-recombination pathway is likely, to explain the transfer of a hydroxyl group from C2 to C1, although a protonated radical species could undergo a concerted hydroxyl migration, as shown by the theoretical studies of Golding and Radom[93].

In summary, the key feature of the hypothesis is that the coenzyme, containing a weak Co-C bond (BDE = 120 kJ mol^{-1}), serves as a source of $RCH_2^{\bullet}$ radicals. The $RCH_2^{\bullet}$ removes a hydrogen atom from the substrate, and it is the protein-substrate interactions that control the subsequent reactions, rather than the coenzyme. The resulting 5'-CH_3 of 5'-deoxyadenosine could be interposed between the substrate-derived radical and the cobalt atom to prevent the unwanted combination. This is in agreement with the 10 - 12 Å distance between the radicals found in the ESR studies[81], and shows how the required transformations can take place with very little relative movement of the reacting species. Proponents of mechanisms that involve organocobalt intermediates must explain how the adenosyl methyl group can swing out of the way to allow the substrate radical to move at least 8 Å, to form a cobalt-carbon σ-bond.

The 'bound radical' mechanism is discussed further in Chapter 6, where the findings of the present work are shown to be consistent with this hypothesis.

Although the evidence discussed above is mostly concerned with the mechanism of diol dehydratase, the hypothesis can be applied

to other adenosylcobalamin-dependent enzymes. It can be used almost without modification for the reaction of ethanolamine ammonia lyase[53], where analogous radical fragmentations are known (Equation 1.15).

Equation 1.15

$$H_2N\text{-}CH_2\text{-}\dot{C}HOH \rightarrow NH_3 + \bullet CH_2\text{-}CHO$$

α-Methyleneglutarate mutase may also be included, since (as mentioned above) butenyl radicals rapidly interconvert with cyclopropylmethyl radicals[90], and cobalt participation is not required. Some workers[107], however, suggest on the basis of model studies that cobalt-carbon σ-bonded species are intermediates.

Methylmalonyl-CoA mutase may use a different mechanism, as several studies suggest cobalt participation in the rearrangement step. It may also include a reduction to give an anionic, cobalt-stabilised species. The model studies[85,86,87] are supported by the failure of ESR studies to show radical intermediates in the methylmalonyl-CoA mutase reaction[80], but these observations do not exclude a very low stationary concentration of radical species.

1.8 Aims of the project

A large number of model studies have been carried out using cobaloximes, and in view of the differences between cobaloximes and cobalamins (section 1.6), we decided to try to extend these studies to cobalamins, which are obviously closer models of the natural enzymic systems.

The enzyme chosen was diol dehydratase, which is probably the

best studied vitamin-B_{12}-dependent enzyme, and the models used were dihydroxyalkylcobalamins, since the corresponding dihydroxyalkylcobaloximes had already been extensively studied by Golding *et al.*[31,56,106], and had been shown to model the regiospecific hydrogen abstraction from C1 of a 1,2-diol, and the subsequent dehydration to an aldehyde.

The techniques used to synthesise, purify and characterise the alkylcobalamins are, however, also relevant to the study of other vitamin-B_{12}-dependent enzymic reactions, since these studies are often marred by the poor characterisation of the alkylcobalamins used. It was hoped that high field NMR spectroscopy could be used for the characterisation of alkylcobalamins, and that diastereoisomeric pairs of cobalamins (differing only in the configuration at one carbon on the alkyl ligand) could be distinguished by this technique. Chapter 4 describes how both of these hopes were fulfilled.

The X-ray crystal structure of only one alkylcobalamin had been determined previously[33]. This was the coenzyme, adenosylcobalamin, which has a very large Co-C-C bond angle (125 °) and a Co-C bond length of 2.05 Å. In view of the importance of the cobalt-carbon bond in the catalytic cycle of adenosylcobalamin-dependent reactions, and the possible intermediacy of other alkylcobalamins, we were interested to find out whether this was a typical bond angle and bond length for the alkylcobalamins. Therefore, the crystal structures of (*R*)- and (*S*)-dihydroxypropylcobalamin were determined (Chapter 5).

The reactions under various conditions of a range of dihydroxyalkylcobalamins were investigated (Chapter 6). They differed in

significant ways from the corresponding dihydroxyalkylcobaloximes, but these differences were consistent with the proposed mechanism of diol dehydratase.

The synthesis of a new dihydroxyalkylcobalamin unable to undergo β-elimination was also started. Although the alkylating agent was prepared, it was not pure, for reasons discussed in Chapter 2, and the resulting alkylcobalamin was therefore also a mixture. Work on this problem had to be stopped at this stage, due to lack of time.

CHAPTER 2

SYNTHESIS OF ALKYLATING AGENTS

CHAPTER 2

SYNTHESIS OF ALKYLATING AGENTS

2.1 Introduction

Following the work of Golding *et al.*[89], *vic*-dihydroxyalkylcobalamins were chosen as model compounds for the investigation of the mechanism of the rearrangement catalysed by diol dehydratase (Scheme 1.1, Chapter 1). It had been shown[31] that 4,5-dihydroxypentylcobaloxime gave pentanal, among other products, when photolysed in the presence of acid (Scheme 6.1, Chapter 6), but the corresponding alkylcobalamins had not been studied.

The use of alkylcobalamins as models, instead of cobaloximes or other achiral cobalt complexes, meant that they had to be synthesised from optically pure alkylating agents, if a mixture of diastereoisomers was not to be produced. Reaction of cobaloxime(I) with a racemic alkylating agent gives two enantiomeric alkylcobaloximes, with identical chemical and physical properties (with the exception of sign of rotation), but reaction of the chiral cobalamin(I) with a similar racemate may give rise to a mixture of two diastereoisomers, possibly in unequal proportion, and with differing properties. Previous workers in this field did not seem to have taken account of this fact, and so no information was available as to whether isomers differing only in the chiral centre of the β-ligand could be distinguished, or perhaps even separated. The differences between diastereomeric cobalamins are discussed in section 3.3, as well as in chapters 4 and 5 on the spectroscopic properties and the X-ray crystal structures of cobalamins, respectively.

Therefore, in synthesising the alkylating agents, the routes chosen had to allow them to be made in optically pure forms.

There are several strategies for synthesising enantiomerically pure compounds, summarized as follows:

i) synthesis of the racemate, with a resolution step at some stage,

ii) synthesis using available natural starting materials with the correct configuration at the chiral centres,

iii) synthesis using chiral reagents which impart the correct chirality to the product.

All these methods have certain advantages and disadvantages. The first method results in the loss of half the product, unless the chiral centre can be epimerised and the product recycled. In any case resolutions can be difficult as they depend on finding a salt or a derivative in which the two diastereoisomers differ sufficiently for them to be separated.

The second method is useful if a suitable chiral starting material can be found. There are some limitations on the reactions that can be used in the synthesis, as they must not racemise the chiral centre. Each reagent must either leave the chiral centre untouched, or cleanly invert it.

The third method ('transfer of chirality') is finding increasing use in organic synthesis as more and more chiral reagents are developed[94]. The enantiomeric excess in these reactions however, is often not satisfactory. Chiral reagents which have been in use for a long time are, of course, enzymes[108], and these usually give good enantiomeric purity.

The second method (the use of the 'chiral pool')[109] is the one used mostly in the present work.

A series of *vic*-dihydroxyalkylcobalamins was synthesised from the corresponding tosylates or halides. This chapter describes the

preparation of these alkylating agents.

The enantioselectivity of cobalamin(I) towards racemic alkylating agents was also investigated, and, to this end, a series of epoxides was synthesised. A preliminary study of the application of FT-IR spectroscopy to alkylcobalamins necessitated the synthesis of [1,1-$^{2}H_{2}$]-ethanol *O*-tosylate.

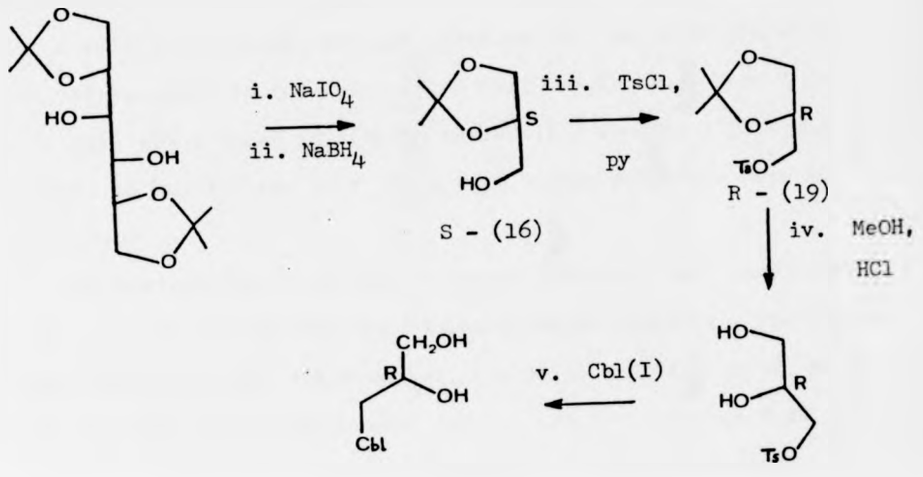
2.2.1 (*R*)- and (*S*)-Glycerol 1-*O*-tosylate

The simplest alkyl group that would give rise to a *vic*-diol on a primary alkylcobalamin is the 2,3-dihydroxypropyl group. This was studied first because it has the chiral centre of the alkyl ligand near to the cobalt atom, so it was anticipated that the differences between the diastereomeric cobalamins would be easiest to detect.

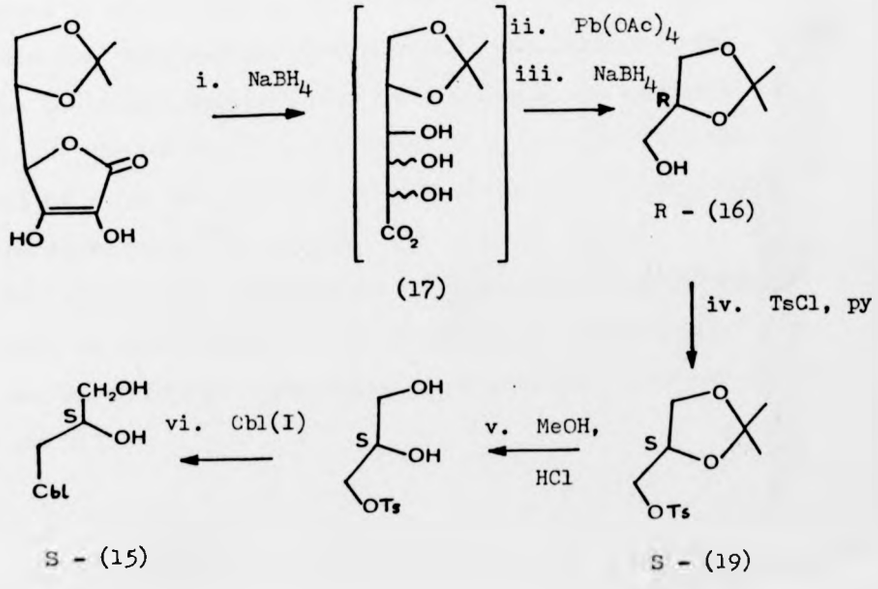
The two enantiomers of glycerol 1-*O*-tosylate were synthesised from compounds in the 'chiral pool', that is, readily available natural compounds having a chiral centre of the correct configuration. (*R*)-2,3-Dihydroxypropylcobalamin ((*R*)-15) was derived ultimately from D-mannitol (Scheme 2.1), while the (*S*)-isomer ((*S*)-15) was made from L-ascorbic acid (Scheme 2.2).

The use of 1,2;5,6-di-*O*-isopropylidene-D-mannitol for the preparation of (*S*)-1,2-*O*-isopropylideneglycerol was first reported by Baer and Fischer[110], who used lead tetraacetate to cleave the protected mannitol into two molecules of (*R*)-2,3-*O*-isopropylideneglyceraldehyde. Borohydride reduction leads to the protected (*S*)-glycerol ((*S*)-16). A modification of the method by Lecoq and Ballou[111] using periodate allowed the use of a less toxic reagent, and the reduction could be carried out without the isolation of the intermediate aldehyde.

Scheme 2.1



Scheme 2.2



(*R*)-1,2-*O*-Isopropylideneglycerol ((*R*)-16) was prepared in an analogous way[112] from L-ascorbic acid protected as the 5,6-*O*-isopropylidene derivative (Scheme 2.2). Reduction with borohydride gave a salt (17) which, without purification, was oxidised with lead tetraacetate to (*S*)-2,3-*O*-isopropylideneglyceraldehyde ((*S*)-18). This was reduced *in situ* to the desired alcohol ((*R*)- 16). Attempts to oxidise the salt (17) with sodium periodate were not successful.

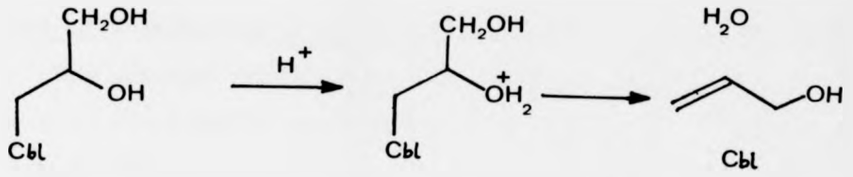
The optical purity of the protected glycerols was assessed by polarimetry and by the use of chiral shift reagents. The (*S*)-isomer had an optical rotation $[\alpha]_D$ = + 11.85 ± 1 °(c = 0.40, MeOH), while the (*R*)-isomer had a value $[\alpha]_D$ = - 10.9 ± 1 ° (c = 0.10, MeOH). The literature value[112] for the (*R*)-isomer is $[\alpha]_D$ = - 10.8 (c = 0.17, MeOH).

The use of the chiral shift reagent, europium(D-3-heptafluorobutyrylcamphorate)$_3$ resolved the diastereotopic *gem*-dimethyl groups but showed no sign of the opposite enantiomer in either case. It is concluded that the enantiomeric excess is > 95 % in each case.

The protected glycerols were converted into the tosylates ((*R*) and (*S*)-19) by the use of tosyl chloride in pyridine[56]. The optical rotation of ((*R*)- 19) was $[\alpha]_D$ = - 4.8 ± 0.5 ° (c = 0.403, EtOH). The literature value[113] is $[\alpha]_D$ = - 4.5 ° (c = 1, EtOH).

The acetal was hydrolysed before alkylation of the cobalamin, to avoid subjecting the β-hydroxyalkylcobalamins to acid, as it was expected that elimination would then take place very easily (Equation 2.1).

Equation 2.1



The tosylates ((*R*) and (*S*)- 19) could be used either directly for alkylation (following hydrolysis of the acetal) or they could be converted into the iodides by the action of sodium iodide in acetone, the Finkelstein reaction.

2.2.2 (*S*)-Butane-1,3,4-triol 1-*O*-tosylate

A sample of (*S*)-4,-(2-hydroxyethyl)-2,2-dimethyl-1,3-dioxolan ((*S*)- 20), with specific rotation in agreement with the literature value[114a] of $[\alpha]_D$ = + 2.2 (c = 0.98, MeOH), was kindly donated by M.H. Brookes[114b] and was tosylated by standard methods 56. The enantiomeric purity was checked by the use of a chiral shift reagent. None of the enantioner was detectable in the 220 MHz ^1H NMR spectrum when europium(D-3-heptafluorobutyrylcamphorate)$_3$ was added, showing that there could not be more than 5 % of the (*R*)-isomer.

The racemic compound ((*RS*)- 21) was prepared from (*rac*)-1,2,-4-butanetriol by protection with acetone and tosylation. Addition of 10 mg of the chiral shift reagent to an NMR sample clearly showed resolution of the two enantiomers.

The acetal could be hydrolysed before alkylation of cobalamin(I), by treatment of the acetal with 5 % acetyl chloride in methanol. Alternatively the protected alkylating agent could be used, and

the acetal hydrolysed subsequently (Method A(ii) Chapter 3)

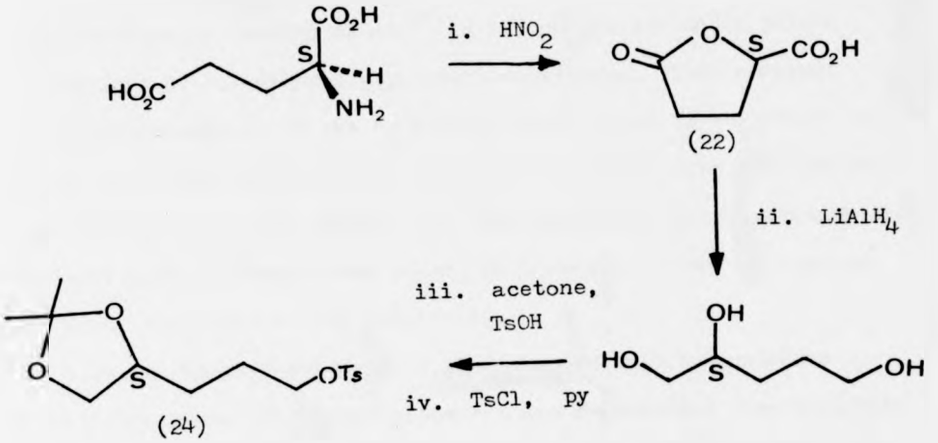
2.2.3 (*S*)-4-(1-Hydroxypropyl)-2,2-dimethyl-1,3-dioxolan 1-*O*-tosylate ((*S*)- 24)

This compound was prepared from L-glutamic acid (Scheme 2.3). The first step, that of replacing the amino group with an oxygen function, followed the method of Ravid *et al*.[115]. This was achieved *via* the diazo compound which spontaneously decomposes to the lactone ((*S*)- 22) with retention of configuration. The optical rotation of the lactone ($[\alpha]_D$ = + 16.0 ± 1 ° (c = 0.02, EtOH)) was in agreement with the literature[115] value (+ 15.6 ° (c = 0.02, EtOH)). The lactone was reduced using lithium aluminium hydride to (*S*)-pentane-1, 2,5-triol (specific rotation $[\alpha]_D$ = - 12.1 ± 1 ° (c = 0.04, EtOH), compared to the literature value[115] $[\alpha]_D$ = - 11.6 ° (c = 0.04, EtOH)). Protection as the acetal of acetone was accomplished by stirring with acetone in the presence of a catalytic amount of *p*-toluene-sulphonic acid. The triol is very hygroscopic, and so some water remains in it even after drying, and is present in the reaction mixture. Therefore the protection with acetone proceeds in low yield. This yield can be increased by recovering the unreacted triol and repeating the reaction.

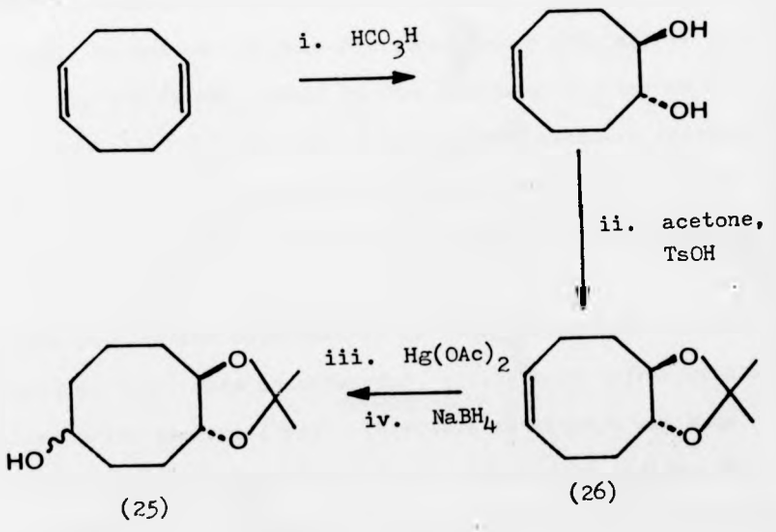
The alcohol ((*S*)- 23) was tosylated in the usual way, and used directly for alkylation of cobalamin(I). The tosylate ((*S*)- 24) was checked for enantiomeric purity by the use of a chiral shift reagent, and none of the opposite enantiomer was detected, showing that the main enantiomer must account for > 95 % of the total.

The racemic compound ((*RS*)- 24) was prepared from (*rac*)-pentane-1,2,5-triol by protection with acetone and tosylation. The enantiomers could be distinguished by the use of the chiral shift reagent.

Scheme 2.3



Scheme 2.4



2.3 Dihydroxycyclooctyl derivatives

A model for diol dehydratase-catalysed rearrangements which has been studied by Golding *et al.*[56] is the photolysis under acidic conditions of 4,5-dihydroxycyclooctylcobaloxime. This compound undergoes homolysis of the Co-C bond, which leads to the formation of an alkyl radical which can abstract a hydrogen atom from across the ring (Scheme 1.3, Chapter 1). The resulting 1,2-dihydroxycyclooctyl radical gives cyclooctanone in *ca.* 40 % yield. It was of interest to study this system with cobalamins.

10,10-Dimethyl-4-hydroxy-9,11-dioxabicyclo[6.3.0]undecane (25) (a mixture of diastereoisomers) was synthesised from *cis, cis*-cycloocta-1,5-diene by the method of Golding *et al.*[56] (Scheme 2.4), and was converted into the mixture of tosylates by standard methods. Separation of the diastereoisomers by fractional crystallisation led to only a small amount of one pure diastereoisomer (240 mg, 14 %) (identifiable by the ^{1}H NMR signal of the proton on the carbon bearing the tosyl group). A 4 : 1 mixture of the diastereoisomers (enriched in the other isomer) was recovered from the mother liquors. Some elimination also took place to give 10,10-dimethyl-9,11-dioxabicylo-[6.3.0]undec-4-ene (26).

Attempts to alkylate cobalamin(I) or hydridocobalamin with either of the tosylates (27) were unsuccessful, giving only hydroxocobalamin and the elimination product (26). Therefore an attempt was made to convert the tosylate mixture into the corresponding iodides, by means of the Finkelstein reaction. This reaction was not successful as it led to extensive elimination giving the olefin (26).

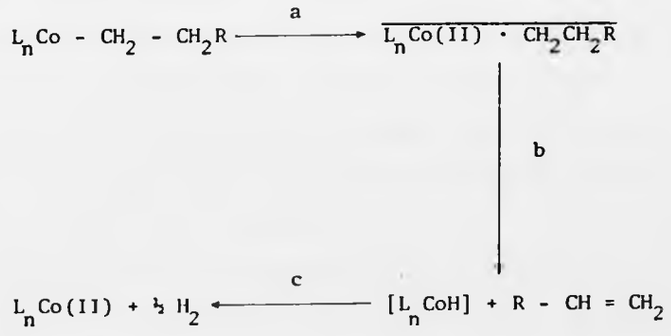
Trifluoromethane sulphonates ('triflates') are reported[116] to be better leaving groups than tosylates by a factor of 10^{5} , and so it was hoped that the reaction of cobalamin(I) with the hindered cyclooctyl derivative would be possible. Model reactions were first

attempted. n-Hexanol 1-*O*-triflate[117] gave n-hexylcobalamin on reaction with cobalamin(I), $NaBH_4$ being used as the reducing agent. Cyclooctanol *O*-triflate did not give cyclooctylcobalamin on reaction with hydridocobalamin (prepared by the reduction of hydroxocobalamin in glacial acetic acid with zinc dust) although iodocyclooctane did react under these conditions to give cyclooctylcobalamin. Therefore the triflate of (25) was not synthesised, as it was assumed that it would not react with hydridocobalamin either.

2.4 4-(3-Iodo-2,2-dimethylpropyl)-2,2-dimethyl-1,3-dioxolan (28)

When alkylcobalamins containing β-hydrogen atoms are photolysed or thermolysed, β-elimination of hydrogen is often the predominant reaction (Chapter 6). The products are olefins and hydridocobalamin, which breaks down under the reaction conditions. Halpern *et al.*[55] suggest that this elimination is not concerted, as suggested by Grate and Schrauzer[118], but instead takes place by the initial formation of a geminate radical pair, followed by β-hydrogen transfer (Equation 2.1).

Equation 2.1



Such hydrogen transfer (step b) was shown to be fast in related systems[119], but this does not rule out the concerted mechanism.

Whether β-elimination is *via* a radical or a concerted mechanism, it competes with the intramolecular hydrogen transfer leading to the isomeric radical, which, it is hoped, will model the rearrangements catalysed by vitamin-B_{12}-dependent enzymes. β-Elimination either prevents the alkyl radical being formed, or gives it an alternative means of breakdown. Therefore an alkylcobalamin which could not undergo β-elimination was designed. This was an analogue of 4,5-dihydroxypentylcobalamin, but with methyl groups replacing the two β-hydrogen atoms.

The above alkylating agent (28) was synthesised in racemic form, although the strategy used could be extended to include a resolution step.

A key intermediate in the synthesis was 4-(3-hydroxy-2,2-dimethylpropyl)-2,2-dimethyl-1,3-dioxolan (29) and an attempt was made to make this compound by the direct alkylation of 4-(iodomethyl)-2,2-dimethyl-1,3-dioxolan (30) with the enolate of methylpropanal, followed by reduction (Scheme 2.5). This has the advantage that (30) can be readily prepared in optically active form from the corresponding protected glycerols (Section 2.2.1).

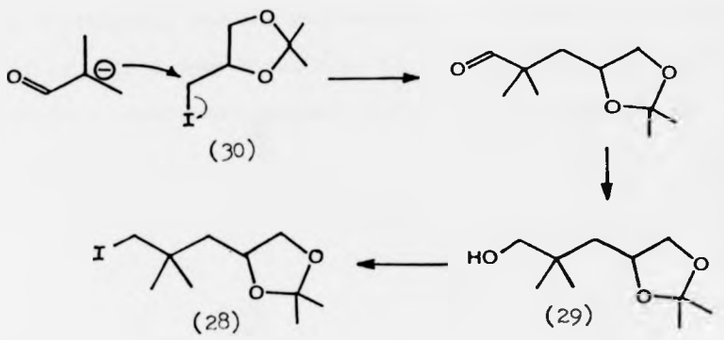
The trimethylsilyl enol ether of methylpropanal was prepared by the method of House *et al.*[120] but treatment with tetrabutylammonium fluoride in the presence of the iodide (30) did not lead to alkylation. This is probably because both the iodide and the enolate are quite sterically hindered.

An alternative strategy was therefore pursued (Scheme 2.6).

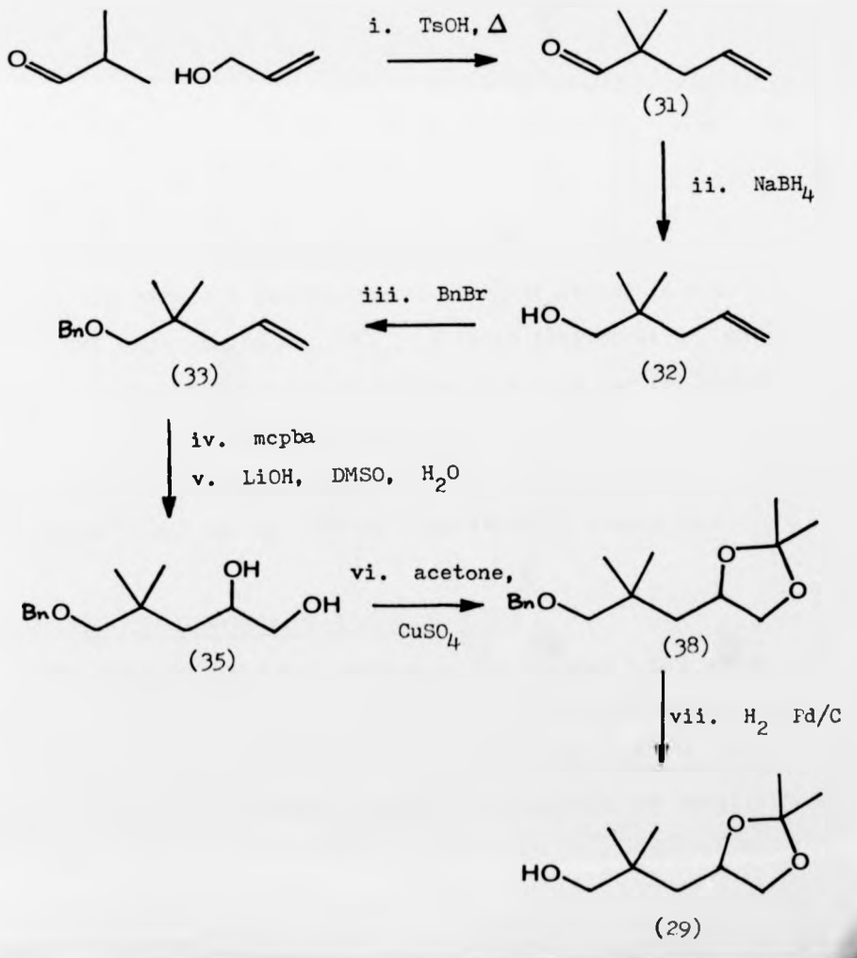
2.4.1 2,2-Dimethylpent-4-enal (31)

This compound was prepared by a Claisen rearrangement using the method of Brannock[121], for making γ,δ-unsaturated aldehydes from vinyl

Scheme 2.5 Projected synthesis of (28)

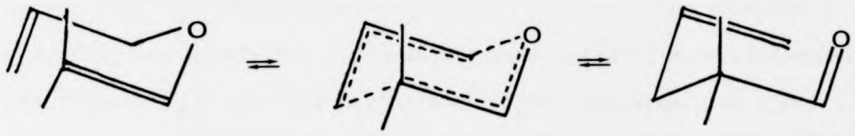


Scheme 2.6 Synthesis of (29)



enol ethers. The enol ether was formed *in situ* from allyl alcohol and isobutanal, and the rearrangement took place on refluxing the mixture with a Dean-Stark trap for the azeotropic removal of water. This is a concerted, thermal [3,3]-sigmatropic mechanism (Scheme 2.7).

Scheme 2.7 Claisen rearrangement



The aldehyde was fractionally distilled out of the reaction mixture. It showed a characteristic aldehyde signal (δ 9.2, s) in its ^{1}H NMR spectrum (60 MHz, CCl_4), a sharp singlet at δ 1.00 (6 H) for the *gem*-dimethyl groups, a doublet at δ 1.15 for the protons on C3, and a multiplet for the three olefinic protons at δ 4.7 - 6.0. The IR spectrum confirmed the presence of an aldehyde, with a peak at 1725 cm^{-1}, and one at 1640 cm^{-1} signifying a double bond.

2.4.2 1-*O*-Benzyl-2,2-dimethylpent-4-en-1-ol (33)

The aldehyde (31) was reduced to the alcohol (32) using sodium borohydride, and was benzylated by the method of Czernecki *et al.*[122]. This involved the use of the phase-transfer catalyst, tetra-n-butylammonium iodide, in order to increase the reactivity of

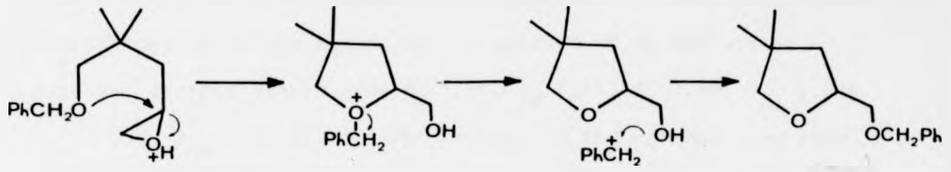
the alkoxide, possibly by the formation of an ion pair ($RO^- \| {}^+NBu_4$). This catalyst allows the use of shorter reaction times and lower temperatures.

The benzyl ether (33) was characterised by its ^1H NMR spectrum (300 MHz, $CDCl_3$), and by its IR and mass spectra. The IR spectrum lacked the hydroxyl peak around 3350 cm^{-1} in the spectrum of the alcohol (32), and instead had peaks at 1500 and 1590 (w) cm^{-1} signifying the presence of an aromatic ring. A peak at 1640 cm^{-1} showed that the double bond was present. The mass spectrum had a molecular ion at m/z 204, with an accurate measured mass confirming the formula $C_{14}H_{20}O$. The high field ^1H NMR spectrum gave the expected signals. The *gem*-dimethyl groups gave a singlet at δ 0.90 and the protons on C3 gave a signal at δ 2.05 (2H, ddd, J_{vic} = 7.5 Hz, $J_{allylic}$ = 1.0, 1.0 Hz). A singlet at δ 3.13 corresponded to the protons on C1, while the olefinic protons had resonances between δ 4.96 and 5.86. The benzyl group gave a singlet at δ 4.50 (the benzylic protons) and a narrow multiplet at δ 7.3 (the aromatic protons).

2.4.3 1-*O*-Benzyl-2,2-dimethylpentane-1,4,5-triol (35)

Hydroxylation of the double bond of (33) presented some problems. Direct hydroxylation using performic acid[123] was attempted but only by-products were obtained. It seems that the first intermediate in this scheme, the epoxide, was very acid-sensitive, and in the presence of formic acid, an intramolecular ring opening occurred to give the substituted tetrahydrofuran (36) by a mechanism such as that in Scheme 2.8.

Scheme 2.8 Possible formation of (36)



It was decided to isolate the epoxide (1-*O*-benzyl-4,5-epoxy-2, 2-dimethylpentane-1-ol) (34)and attempt a base-catalysed ring opening. The reagent chosen to make the epoxide was *m*-chloroperbenzoic acid, which is less acidic than performic or peracetic acid, and so should allow isolation of the product. Preliminary experiments in which the reaction mixture was left at - 20 ° overnight, led to very little epoxide being isolated, and a large amount of the tetrahydrofuran derivative and other impurities.

Careful monitoring by ^{1}H NMR (60 MHz) showed that the epoxide was formed rapidly at room temperature and the olefin signals could be seen becoming less intense. Before the olefin had completely disappeared, however, a new set of signals began to appear (described below), showing that the epoxide was being consumed in a side reaction. Therefore the reaction was stopped when the decreasing olefin concentration was approximately equal to that of the unwanted product. The epoxide (34) could then be isolated in about 60 % yield. Column chromatography separated the epoxide from the unreacted olefin and from two impurities which were later identified. The epoxide (34) was characterised by ^{1}H NMR and by m.s.

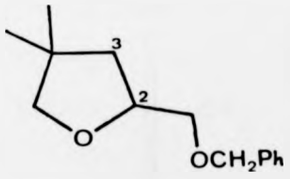
The introduction of a chiral centre meant that the enantiotopic groups in the precursor became diastereotopic in the epoxide, and therefore were potentially distinguishable by NMR spectroscopy.

This was not seen in the case of the *gem*-dimethyl group (δ 1.02, 6H, s) in the 300 MHz ^{1}H NMR spectrum of the epoxide, but the protons on C3 now formed the AB portion of an ABX system. Analysis of this system gave δ_A 1.50, δ_B 1.55, δ_X 2.97, J_{AX} = 6.4, J_{BX} = 5.3, J_{AB} = 14.12 Hz. The protons on the oxirane ring gave three separate signals all coupled, at δ 2.40, 2.70 and 2.97. The signal at δ 2.97 was assigned to the proton on C4 as it was also coupled to the proton on C3. The protons on C1 gave an AB system, δ_A 3.19, δ_B 3.25, J_{AB} = 8.8 Hz, but the benzylic protons were not distinguishable, giving a singlet at δ 4.51. The aromatic protons were at δ 7.2 - 7.4.

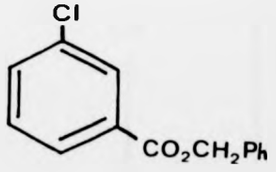
The mass spectrum of the epoxide had a molecular ion at m/z 220, with an accurate mass confirming $C_{14}H_{20}O_2$ as the formula.

The two impurities were identified by ^{1}H NMR and m.s. to be *O*-benzyl-2-hydroxymethyl-4,4-dimethyltetrahydrofuran (36) and benzyl *m*-chlorobenzoate (37).

(34)



(36)

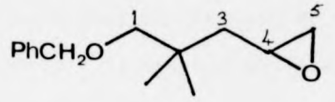


(37)

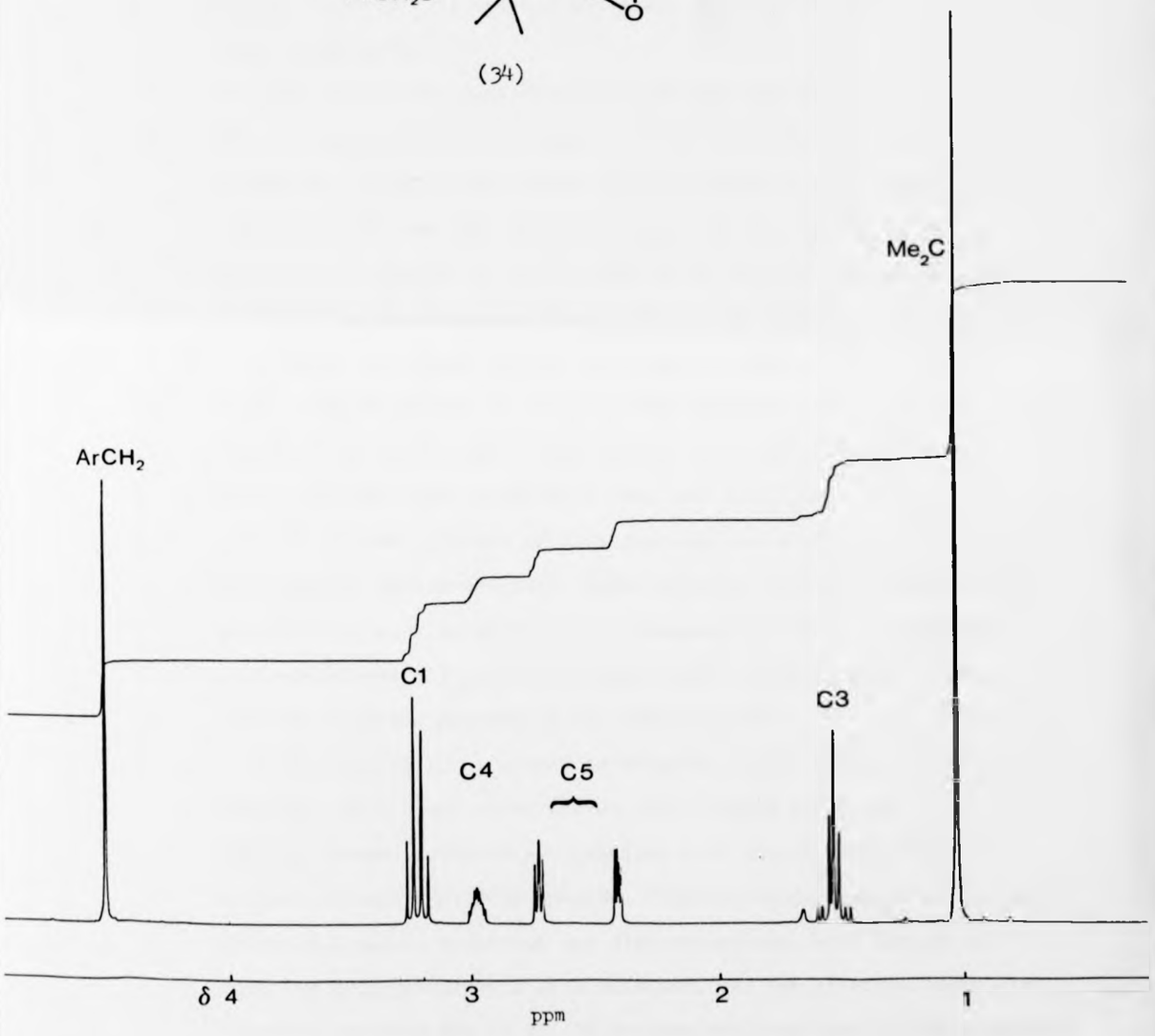
The first by-product (36) had a molecular ion of m/z 220, the same as that of the epoxide, although the fragmentation pattern was different. The 60 MHz ^{1}H NMR spectrum was quite different from that of the epoxide, having none of the signals between δ 2 and 3

^{1}H NMR spectrum of (34) (300 MHz, $CDCl_3$)

(aromatic protons not shown)



(34)



characteristic of the protons on the oxirane ring, but instead having signals integrating to 3H between δ 3 and 4.5, corresponding to the protons on C2 and C2'. The diastereotopic protons on C3 gave an AB portion of an ABX system centred at δ 1.6, while the corresponding signal in the epoxide spectrum (C3 protons) was at higher field (δ 1.4) and had different coupling constants and chemical shift difference.

At 60 MHz, the diastereotopic *gem*-dimethyl groups on C4 were not resolved, and gave a signal at δ 1.05 (6H, s), as opposed to the signal at δ 0.95 for the corresponding groups on the epoxide. The two protons on C5 also gave just one signal at this spectrometer frequency, a singlet at δ 3.3, and the protons of the benzyl group could not be distinguished from those of the epoxide.

Benzyl *m*-chlorobenzoate (37) had a molecular ion at m/z 246, with a characteristic chlorine isotope pattern. There were also fragments corresponding to the benzyl group (m/z 91, $C_7H_7^+$) and to the *m*-chlorobenzoate group (m/z 139, 141, $ClC_6H_4CO^+$).

The ^1H NMR spectrum of this compound had a signal at δ 5.37, for the benzylic methylene group, quite distinct from the corresponding group in the epoxide which had a resonance at δ 4.52. The other resonances were all in the aromatic region and overlapped with those from the aromatic protons of the epoxide (34).

The epoxide (34) proved to be quite stable towards base-catalysed opening. Four hours under reflux with lithium hydroxide (2 M) in aqueous dioxan, followed by overnight stirring at room temperature did not decompose all the epoxide. Phase-transfer-catalysed hydrolysis using 50 % sodium hydroxide and dichloromethane, with tetrabutylammonium hydrogensulphate as a catalyst, was not effective even with vigorous stirring for 72 h. An attempt was also made to use "anhydrous OH^-",[124] which has been used for various hydrolyses. The hydroxide

ion was produced by adding water to an excess of potassium t-butoxide in dry ether, which produces hydroxide ions solvated by ether, and so likely to be very reactive. The method was successful in the hydrolysis of ethyl benzoate, but when phenyl glycidyl ether was used as a model for the epoxide, some epoxide remained even after 24 h.

The method which was finally successful was the use of lithium hyroxide in 75 % aqueous DMSO, heated to between 110 ° and 130 ° for 6 h. The DMSO was removed from the diol (35) under vacuum at 40 - 60 °. Attempts to extract the DMSO into water led to loss of product as the diol was also extracted.

The diol (1-*O*-benzyl-2,2-dimethylpentane-1,4,5-triol) (35) was purified by column chromatography and its structure confirmed by inspection of the m.s. and ^1H NMR spectrum The mass spectrum had the molecular ion at m/z 238, with measured mass (238.1579) in good agreement with that required for $C_{14}H_{22}O_3$ (238.1569). The ^1H NMR spectrum (300 MHz, $CDCl_3$) showed the loss of epoxide signals between δ 2 and 3, and the appearance of three separate proton signals between δ 3.3 and 3.9, corresponding to the three protons on C4 and C5. The protons on C3 gave two double doublets at δ 1.35 and 1.50, rather than the overlapping ABX system seen in the epoxide. The *gem*-dimethyl groups were quite separate in the diol, at δ 0.93 and δ 1.01. Addition of D_2O caused two broad signals between δ 2 and 3 to disappear, demonstrating the presence of two hydroxyl groups. The signals from the benzyl group were in the same positions as those in the epoxide.

It had been hoped to proceed directly from the epoxide to the acetonide (38) by addition of acetone, as this decreases the number of steps and also saves adding water to the molecule, only to remove it in the next step. There are various Lewis-acid-catalysed conversions

of epoxides to acetals, using for instance, copper(II) sulphate[125], tin(IV) chloride[126] or borontrifluoroetherate[127] as catalysts. The acid-sensivitiy of the epoxide meant that these methods were not practicable. Although propylene oxide was converted in good yield into the corresponding acetonide by BF_3-Et_2O in dry acetone (Scheme 2.9), when the same procedure was tried on the epoxide (34), no acetonide was formed (as shown by m.s. and NMR). When the experiment was done in d_6-acetone and followed by NMR spectroscopy (60 MHz), as soon as the catalyst was added, the epoxide signals disappeared and signals probably corresponding to the tetrahydrofuran derivative (36) were seen. These did not change further. After filtration through K_2CO_3 and evaporation of the acetone, m.s. did not show a molecular ion corresponding to the acetonide, or (M+ - 18) corresponding to M+ - CD_3, although loss of one of the *gem*- methyl groups is characteristic of acetals of acetone. A peak at m/z 220 probably corresponded to (36).

2.4.4 *O*-Benzyl-4-(3-hydroxy-2,2-dimethylpropyl)-2,2-dimethyl-1,3-dioxolan (38)

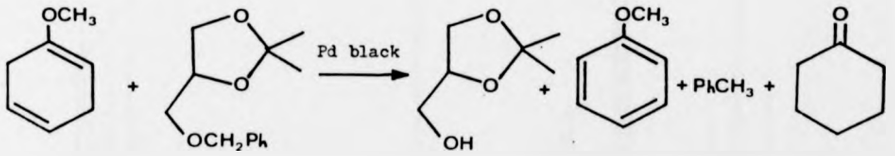
The formation of the isopropylidene derivative (38) of the diol (35) was accomplished by using anhydrous copper(II)sulphate and a catalytic amount of concentrated sulphuric acid in freshly distilled acetone[125] The copper(II)sulphate served both as a drying agent and as a Lewis-acid catalyst. The acetal formed in good yield (> 70 % after chromatography) and was characterised by ^{1}H NMR, IR and mass spectroscopy. The mass spectrum showed a small peak at m/z for the molecular ion, and a much larger peak at m/z 263 corresponding to the facile loss of a methyl group from the acetal. The ^{1}H NMR spectrum included two three-proton singlets from the isopropylidene group (δ 1.33 and 1.38) as well as the singlets from the methyl groups on

C2, at δ 0.96 and 0.97. The other peaks were similar to those in the diol. The IR spectrum showed the absence of hydroxyl groups.

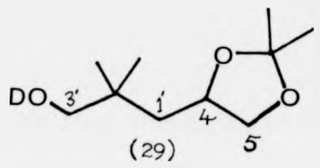
2.4.5 4-(3-Hydroxy-2,2-dimethylpropyl)-2,2-dimethyl-1,3-dioxolan (29)

The above alcohol (29) was formed by the hydrogenation of the benzyl ether (38) by standard methods[128]. An attempt was made to use a modification of a catalytic transfer hydrogenation method employing 1,4-cyclohexadiene as the hydrogen transfer agent and palladium black as the catalyst[129,130]. Since 1,4-cyclohexadiene is quite expensive, it was replaced by 2,5-dihydroanisole which was prepared by Birch reduction of anisole[131]. Using 1,2-*O*-isopropylidene-3-*O*-benzylglycerol (39) as a model for the benzyl ether (38), the hydrogenation was attempted (Equation 2.2). On work-up of the reaction mixture, it was seen that no dihydroanisole remained, although not all the benzyl ether had reacted. The main products were anisole and cyclohexanone, that is, the main reaction was the transfer of hydrogen between molecules of dihydroanisole. The same result was obtained if the benzyl ether was left out of the reaction mixture. In this case anisole and cyclohexanone were the only products (Equation 2.3).

Equation 2.2

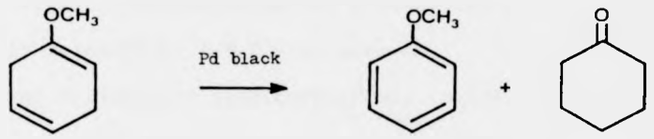


^{1}H NMR spectrum of (29) (300 MHz, $CDCl_3$)



(29)

Equation 2.3



It was found that hydrogenation using hydrogen at atmospheric pressure over a 10 % Pd/C catalyst led to rapid uptake of the stoichometric amount of hydrogen, and a clean conversion of the benzyl ether (38) to the alcohol (29). The alcohol was characterised by m.s., IR and ^{1}H NMR spectroscopy. No molecular ion was visible in the m.s., but the highest mass peak was at m/z 173 (M+ - 15) characteristic of the loss of a methyl group from an acetal of acetone. The ^{1}H NMR spectrum also showed the complete loss of the benzyl group, and the IR spectrum showed a clear hydroxyl signal at 3420 cm^{-1}.

2.4.6 4-(3-Iodo-2,2-dimethylpropyl)-2,2-dimethyl-1,3-dioxolan (28)

Conversion of the alcohol (29) into the iodide (28) was expected to present some problems as neopentyl-type alcohols have a tendency to undergo acid-catalysed rearrangements[132]. Neopentyl iodide itself was made[133] by treating neopentyl alcohol with triphenyl phosphite and methyl iodide with prolonged heating at 140 °. These seemed harsh conditions and so a milder method was looked for.

The Mitsonobu reaction[134] was investigated, as it should be a mild method for the conversion of primary alcohols into iodides. In this reaction, a salt is formed by the reaction of a dialkyl azodicarboxylate and triphenylphosphine, which activates alcohols towards nucleophilic displacement *via* the formation of an alcohol-salt

complex. Methyl iodide can be used as a source of iodide ions (Scheme 2.10). Triphenylphosphine attacks the methyl iodide, and iodide is released by a S_N2 displacement.

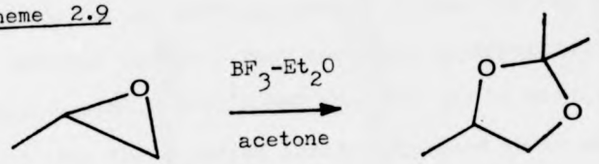
Using diisopropyl azodicarboxylate (DIAD), triphenylphosphine, and methyl iodide, the conversion was attempted of 1,2-*O*-isopropylideneglycerol (16) into its iodide. Although triphenylphosphine oxide was produced, the starting alcohol was recovered unchanged.

A method which had previously been used for the conversion of 1,2-*O*-isopropylideneglycerol into its iodide was the use of triphenoxymethylphosphonium iodide[135]. The alcohol was heated with the phosphonium salt under anhydrous conditions for 6 h at 50 °, and the iodide was produced in 75 % yield. The reagent was essentially the same as that used to convert neopentyl alcohol into its iodide, but in this case the methiodide was already formed, as the reaction proceeded at a much lower temperature (Scheme 2.11). Later modifications[136] of this method used dimethylformamide (DMF) as a solvent, and included pyridine to neutralise any hydrogen iodide produced by the action of traces of water on the phosphonium salt.

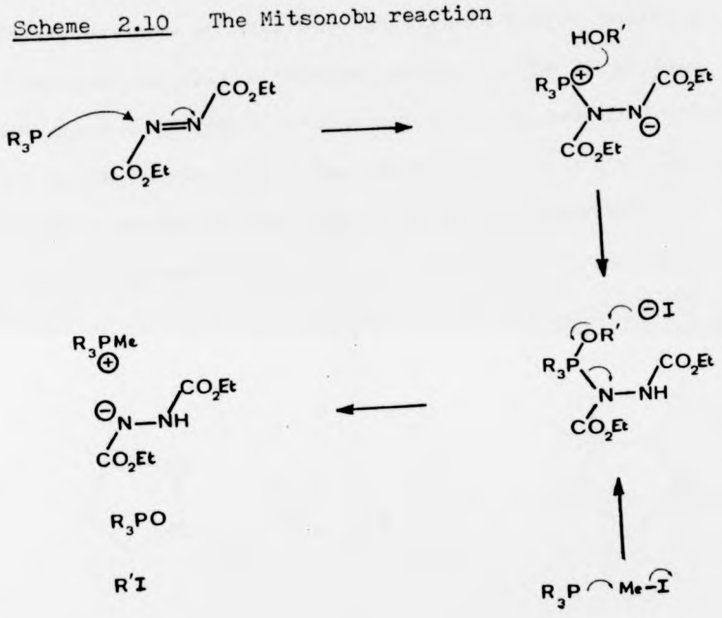
The use of a mixture of benzene and DMF as a solvent, and 10 mol % of pyridine, was found to be very successful in converting (16) into its iodide, which was produced in 2 h at room temperature. ^1H NMR monitoring suggested that the reaction was probably complete within 15 min. The iodide (30) was isolated in 82 % yield after chromatography. The method was used on neopentyl alcohol, and this also showed complete conversion into its iodide within a few minutes, with no evidence of rearrangement.

When this method was used on 4-(3-hydroxy-2,2-dimethylpropyl)-2,2-dimethyl-1,3-dioxolan (29), the starting material was rapidly

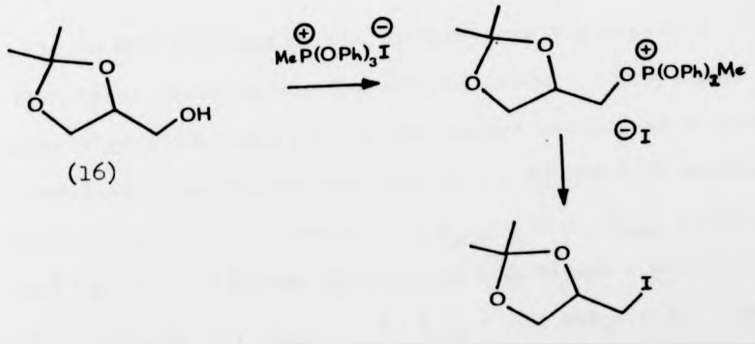
Scheme 2.9



Scheme 2.10 The Mitsonobu reaction



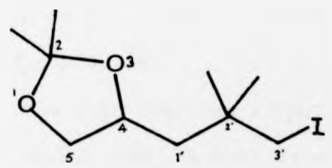
Scheme 2.11



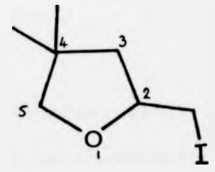
(16)

(30)

consumed, but on work-up, another product was isolated as well as the expected iodide. They could not be separated by flash column chromatography. The ^{1}H NMR spectrum of the mixture (300 MHz, $CDCl_3$) showed that the expected iodide comprised about one third of the total, the rest being the unknown product, apart from < 5 % of another product. The main component lacked the *gem*-dimethyl group of the acetone acetal, but had signals corresponding to all the other protons in the expected product. The AB portion of an ABX system was centred around δ 3.25, which is at higher field than that expected for a -CH-CH_2-O- signal but characteristic of a -CH-CH_2-I signal. For this reason it was thought to be 2-iodomethyl-4,4-dimethyl-tetrahydrofuran (40).



(28)



(40)

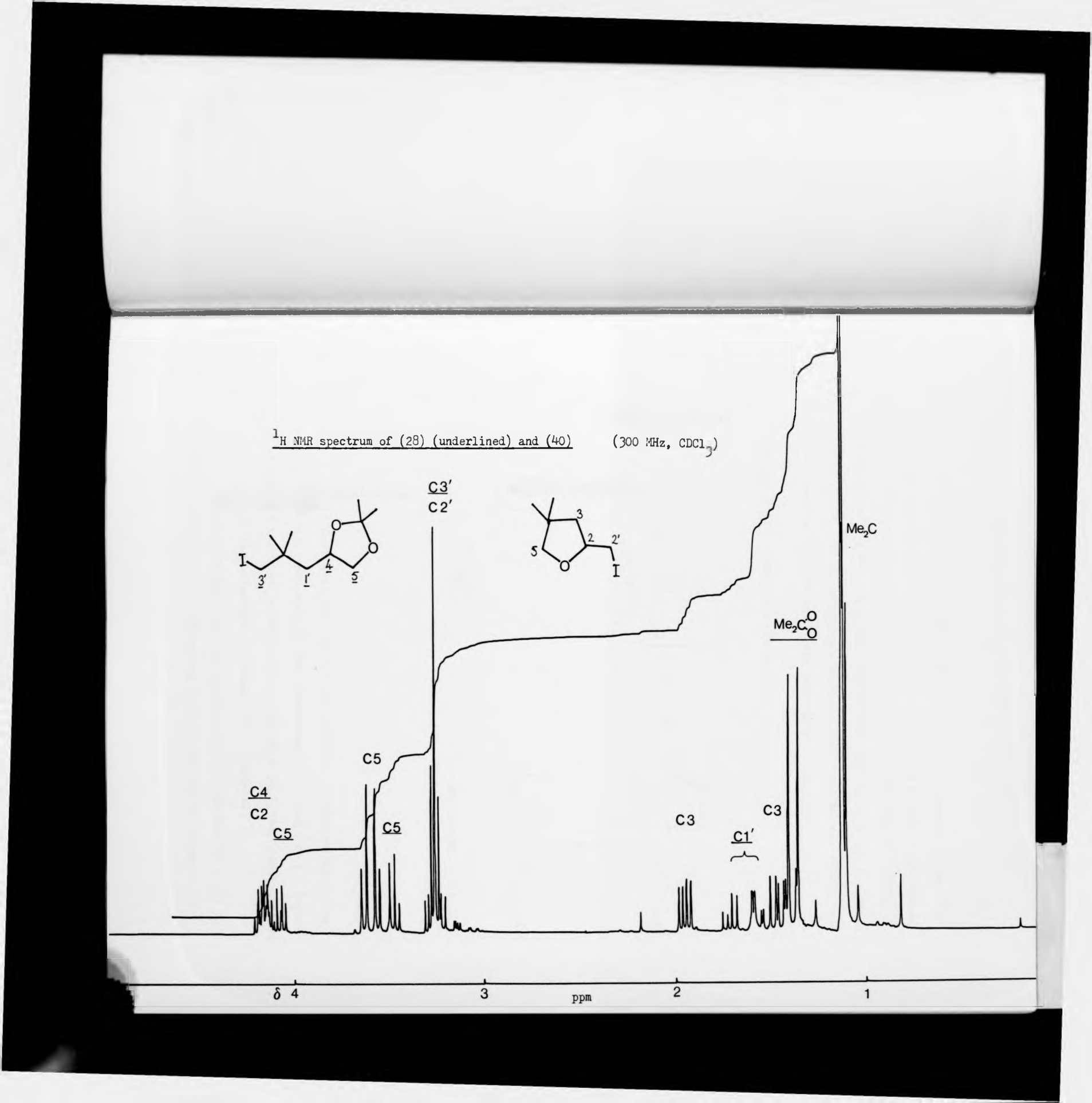
The ^{1}H NMR spectrum of the mixture gave the expected signals for both these structures. The desired product (28) showed a separate signal for each of the four methyl groups, at δ 1.08, 1.11, 1.35 and 1.40. Two double doublets at δ 1.55 and 1.70 corresponded to the diastereotopic protons on C1', J_{gem} = 14.4, J_{vic} = 3.9 and 8.0 Hz, and two further double doublets at δ 3.46 and 4.06 corresponded to the protons on C5, J_{gem} = 7.7, J_{vic} = 7.7 and 5.9 Hz. The C4

hydrogen atom gave a complex multiplet at δ 4.13, overlapping with a multiplet from the other product. The protons on C3′, which carried the iodine atom, should have given an AB system. Integration of the signal centred at δ 3.25 suggested that the signal from the C3′ - H_2 was hidden beneath the AB portion of an ABX system from the other compound.

The tetrahydrofuran derivative (40), which comprised two thirds of the mixture, gave a somewhat similar spectrum. The *gem*-dimethyl group gave two separate signals at δ 1.10 and 1.12, and the protons on C3 gave two double doublets at δ 1.45 and 1.94, J_{gem} = 12.5 Hz, J_{vic} = 8.7 and 6.6 Hz. An AB system for the C5 protons was seen with δ_A = 3.55, $\delta_{\bar{B}}$ = 3.62, $J_{A\bar{B}}$ = 8.1 Hz and the proton on C2 gave a complex multiplet at δ 4.15. The protons on the carbon bearing the iodine atom gave the AB portion of an ABX system (X being the proton on C2), with δ_A = 3.28, δ_B = 3.22 and J_{AB} = 9.9, J_{AX} = 5.4 and J_{BX} = 6.7 Hz.

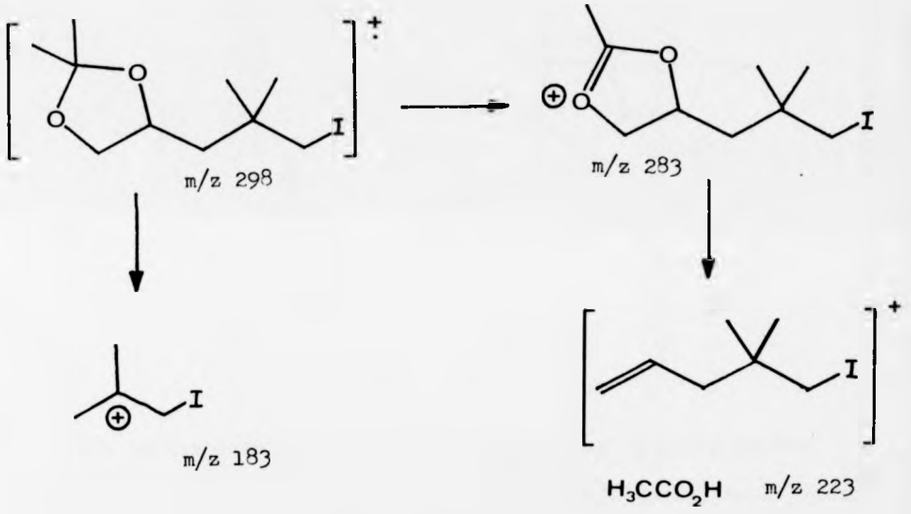
The mass spectrum supported this hypothesis, as it showed a peak at m/z 240, as well as one at m/z 298 which corresponded to the molecular ion of the expected product. A peak at (M+ - 58) could be explained by the loss of acetone from the expected iodide (28), but acetals of acetone do not usually decompose in this way. Therefore it was more likely that the ion at m/z 240 was the molecular ion of the contaminating product (40).

The structure of the iodide (28) was confirmed as follows. As well as the molecular ion at m/z 298, there was a peak of higher intensity at m/z 283, corresponding to the loss of one of the *gem*-dimethyl groups of the acetone acetal. The accurate mass measurements of both these peaks confirmed the molecular formula $C_9H_{16}O_2I$ for the iodide.


1H NMR spectrum of (28) (underlined) and (40) (300 MHz, CDCl3)
C3′
C2′
Me2C
Me2C
O
O
C4
C2
C5
C5
C5
C3
C1′
C3
δ 4
3
ppm
2
1

A peak at m/z 268 (M+ - 30) probably corresponded to the loss of two methyl groups. The peak at m/z 223 was probably associated with the loss of acetic acid from the ion at 283 (Scheme 2.12), particularly since there was a metastable peak at m/z 175.5. As $M^* = M_2^2/M_1$, if M_1 decomposes to M_2 in one step, it was likely that the reaction m/z 283 → 223 was occurring.

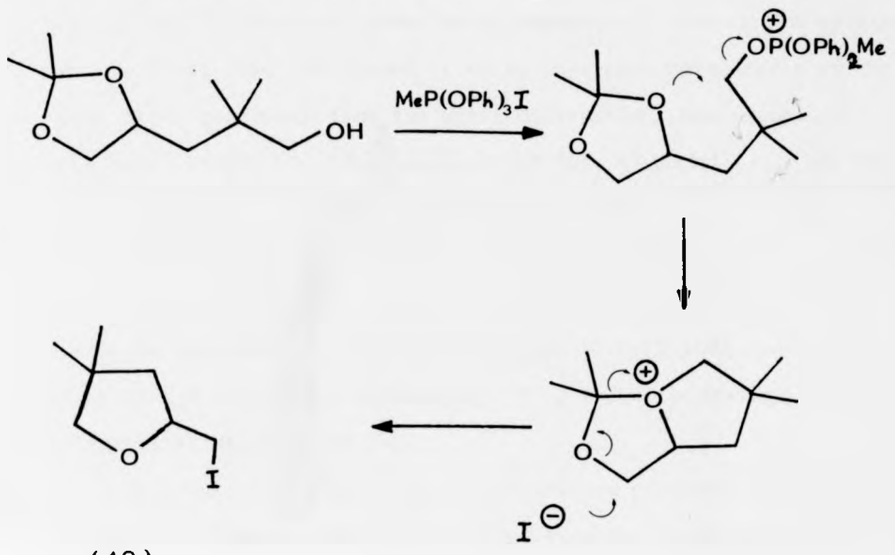
Scheme 2.12 Fragmentation of (28)



There were also ions at m/z 171 and 113, corresponding to the loss of iodide from each of the structures (28) and (40). A peak at m/z 183 probably corresponded to the $Me_2\overset{+}{C}-CH_2I$ ion, demonstrating the one of the structures included this fragment.

It was envisaged that the tetrahydrofuran derivative (40) could have formed by a mechanism such as the one in Scheme 2.13.

Scheme 2.13 Formation of (40)



(40)

The intramolecular reaction is accelerated by the presence of the *gem*-dimethyl group which, for entropic reasons, makes cyclisation easier (the Thorpe-Ingold effect)[137].

It was hoped, therefore, that by increasing the concentration of iodide ions in the reaction mixture, the intermolecular reaction might compete more successfully with the intramolecular one. For this reason, the reaction was repeated with the addition of 5 molar equivalents of tetra-n-butylammonium iodide, an iodide which is soluble in the reaction medium. Work-up, by extraction and flash column chromatography as before, gave a mixture of products.

An attempt was made to separate these by HPLC. An analytical

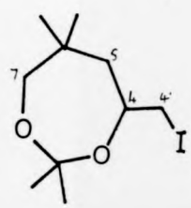
column gave three separate peaks, but a preparative column gave two fractions, the last two peaks being unresolved. The ^1H NMR spectrum of the first fraction showed it to be an almost pure sample of the very minor component from the previous reaction, now present as the major component. The structure of this compound (41) has not been fully elucidated, but certain features of its spectra are described below, and a possible structure is suggested.

The second fraction from the HPLC column was shown by ^1H NMR to be an approximately equimolar mixture of 4-(3-iodo-2,2-dimethylpropyl)-2,2-dimethyl-1,3-dioxolan (28) and 2-(iodomethyl)-4,4-dimethyltetrahydrofuran (40).

Analysis of the spectrum of the unknown product (41) showed features in common with those of the other two components of the mixture. Four singlets were present at δ 0.82, 1.03, 1.36 and 1.41, corresponding to four methyl groups and showing that the isopropylidene group was still present. Two signals, corresponding to one proton each, were also visible at high field, δ 1.55 and approximately 1.3. At lower field, the signals were all between δ 3 and 4. There was a double doublet at δ 3.04, coupled to a doublet at δ 3.65 (J_{gem} = 12.5 Hz). The AB portion of an ABX system was centred at δ 3.14, and there was a complex multiplet (the X proton) at δ 3.95.

The mass spectrum of this compound had a molecular ion at m/z 298, the same as that of the iodide (28). There were also peaks at m/z 283 and 268, corresponding to the loss of one or two methyl groups, respectively. An ion at m/z 223 could correspond to the loss of acetic acid from the ion at m/z 283. Although these peaks had the same masses as those in the spectrum of the iodide (28), the relative intensities were quite different, and, significantly, there was no ion at m/z 183, diagnostic of a $(CH_3)_2\overset{+}{C}CH_2I$ fragment. Therefore

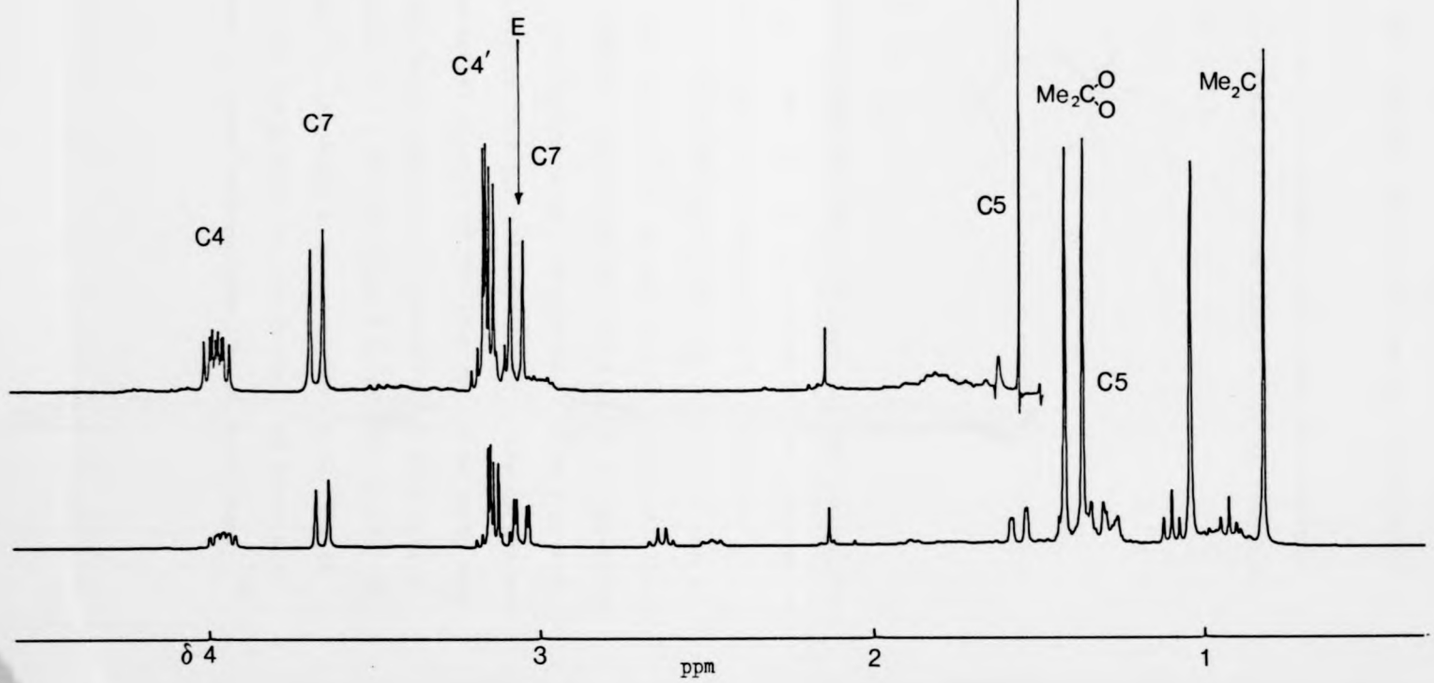
^{1}H NMR spectrum of (41) (300 MHz, $CDCl_3$)



inset: C5 decoupled showing ^4J coupling to C7

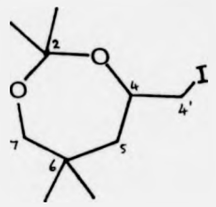
D = irradiation

E = effect on C7



it seemed that in this compound the iodine atom was not attached to a $R\text{-}C(CH_3)_2CH_2$-group.

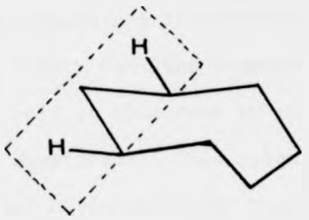
A possible structure, in agreement with these observations, and mechanistically feasible is 4-iodomethyl-2,2,6,6-tetramethyl-1,3-dioxacycloheptane (41).



(41)

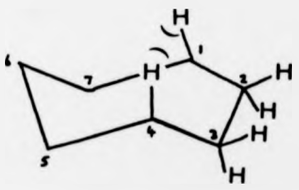
This has four non-equivalent methyl groups, an ABX system comprising the protons on C4 and C4', an AMX system for the protons on C4 (X) and C5 (A and M), and an AB system for C7. The only unexplained feature is the additional splitting observed of one of the C7 protons (δ 3.04, J = 2.3 Hz) to give a double doublet rather than the expected doublet. This might be explained by a long-range coupling to one of the protons on C5. Inspection of the spectrum did not confirm or refute this possibility as one of the C5 protons was obscured by the methyl signals of the isopropylidene group and possibly also by some other impurity. Irradiation of this region showed that the proton at δ 1.54 was coupled to the one on C7. The long range coupling (^4J) is possible in a seven membered ring if the conformation allows the protons to lie in the same plane with a zig-zag arrangement of bonds between them (the W-arrangement)[138] (Scheme 2.14).

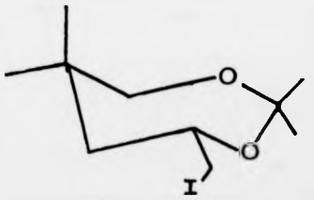
Scheme 2.14 The W-arrangement



This coupling suggests the presence of a cyclic compound in which the substituents are held in the particular conformation. Analysis of cycloheptane[139] shows that it is most stable in a chair-type conformation (Figure 2.1). This is not a strain-free conformation like the chair form of cyclohexane, but has eclipsing interactions between the hydrogens on C2 and C3, and a close approach between the pseudo-axial hydrogens on C1 and C4.

Figure 2.1

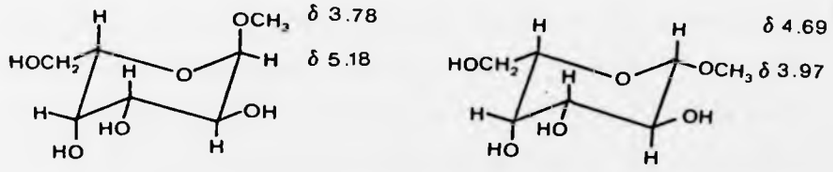




(a)

Both of these unfavourable interactions are removed if C1 and C3 are replaced by oxygen, as in the 1,3-dioxacycloheptane (41), where the lone pairs on oxygen are less sterically demanding than C-H groups. Therefore, it is likely that the compound (41) exists in the chair-like form (a), held in this form by the constraint of having the iodomethyl group equatorial. This allows the pseudo-equatorial hydrogens on C5 and C7 to adopt the W-arrangement, and to show the observed ^{4}J (= 2.3 Hz) coupling. This conformation also suggests a possible explanation of the unusually low value of the chemical shift of one of the *gem*- methyl groups on C6 (δ 0.82). Axial substituents on cyclohexanes or tetrahydropyrans which are fixed in a particular chair form tend to be shielded relative to equatorial substituents[138]. This effect is most marked when hydrogen is the substituent (Δδ = 0.5 ppm), but is observed for the methoxy substituent on α- and β-methoxygalactose (Figure 2.2). The effect is

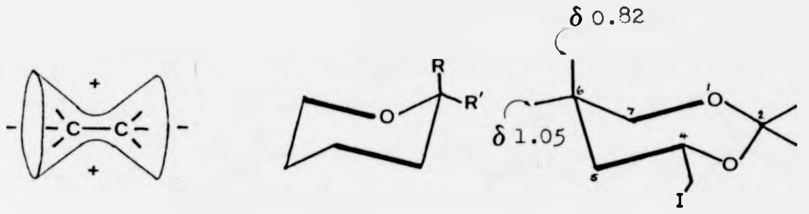
Figure 2.2



produced by the shielding behaviour of the C-C or C-O bonds β-γ to the substituent (Figure 2.3a). The axial substituent lies in the shielded region of these bonds (b), while the equatorial substituent R′, does not. Therefore R is shielded relative to R′, and resonates

at higher field. Thus, if the axial methyl group on C6 of the 1,3-dioxacycloheptane (41) is similarly shielded by the C4 - C5 and C7 - 01 bonds, it will give the observed high field signal (c).

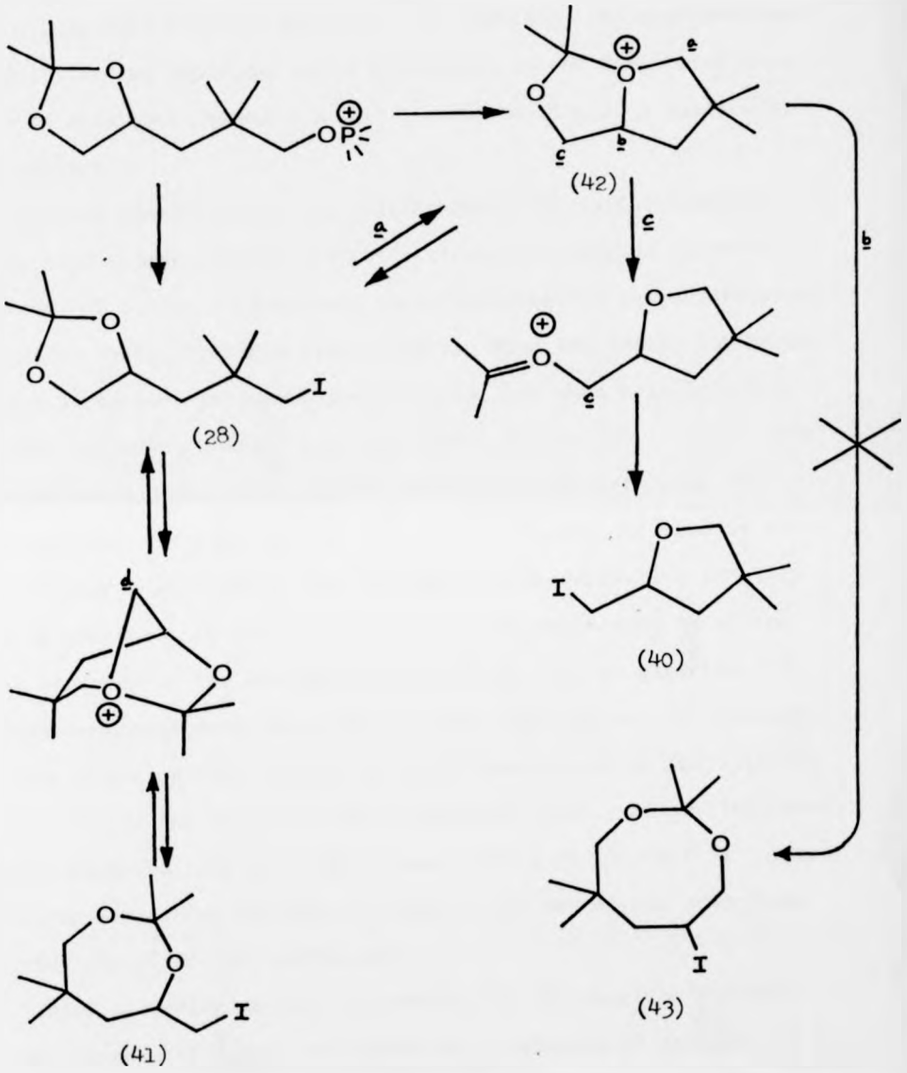
Figure 2.3



a b c

A mechanistic scheme which unifies these findings is outlined in Scheme 2.15. The intermediate (42) is most rapidly formed, a 5-membered ring being kinetically favoured over a 6-membered one. In the presence of iodide ion, the most likely site of attack is at a, the least hindered carbon which is activated towards nucleophilic attack, to give the desired iodide (28). The alternative site of attack of I^- , b, would give rise to the 8-membered ring (43). This product was not observed in the reaction mixtures, unless the product identified as the 1,3-dioxacycloheptane derivative (41), was in fact the 1,3-dioxacyclooctane (43). This was thought to be unlikely for several reasons. Firstly, the cyclooctane derivative contains a proton on the carbon bearing the iodine atom which should give a signal at $\sim \delta$ 4.3, whereas the multiplet is at δ 3.95,

Scheme 2.15



which is more characteristic of a -$\overset{|}{C}$H-O- group. Secondly, the m.s. of (41) shows a peak at m/z 141 (CH_2I^+), which could not be formed by a single bond breakage of (43). In addition, the conformational analysis, above, would not be so applicable to the 8-membered ring, which is less constrained than the 7-membered ring to a particular conformation.

Another possible reaction (c) (Scheme 2.15) is the cleavage of the Me_2C-O bond, leading to the activation of carbon c. When captured by I^-, this intermediate gives the observed tetrahydrofuran derivative (40), probably irreversibly. When the iodide ion is not present in excess, pathway c predominates, but when more iodide is present, pathway a becomes more important, giving (28). Under the reaction conditions, this could be in equilibrium with both the intermediates (42) and (44). If (42) is formed, it reverts to (28) faster than it gives the tetrahydrofuran derivative (40) by loss of acetone. If (44) is formed, it can react with I^- at the least hindered of the activated carbon atoms (d), to give the 1,3-dioxacycloheptane derivative (41). This reaction may be reversible, and the ratios of (40) and (41) would then depend on the relative stabilities of the five- and seven-membered rings. For cycloalkanes, a seven-membered ring is slightly more stable ($\sim$ 2 kJ mol^{-1}) than a five-membered one, but the introduction of two oxygen atoms into the ring may alter this difference.

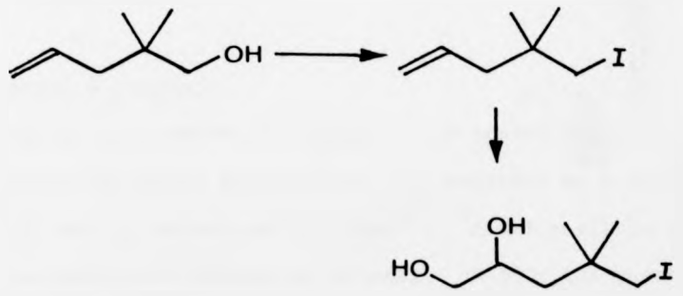
Thus, according to this hypothesis, if the reaction proceeds in the presence of excess of iodide ion, a mixture of iodides (28), (40) and (41) will be produced. The ratio will depend on the concentration of iodide ion, and the relative rates and equilibrium constants involved. This hypothesis is supported by the fact that when the reaction was allowed to proceed in the presence or

absence of excess of iodide, different ratios of (28), (40) and (41) were produced. The reaction could be monitored by HPLC in order to determine the optimum time required to isolate the maximum amount of the iodide (28).

Alternatively, these problems might be overcome by the use of a different iodination agent, but any strategy based on the activation of the hydroxyl group towards nucleophilic displacement is liable to give similar by-products by intramolecular reactions.

Another possible strategy is to choose a route that introduces the halide before the terminal double bond has been functionalised. This strategy is outlined in Scheme 2.16, but lack of time meant that it was not attempted. It is possible that an intramolecular

Scheme 2.16



displacement of iodide could give rise to similar cyclic by-products. The ease of formation of five- and six-membered rings, and the enhancement of the rate of ring-closure by substituents such as the *gem*-dimethyl group (the Thorpe-Ingold effect)[137] mean that this type of compound is likely to undergo such reactions.

2.5 Epoxides

The epoxides (45) and (46) were made in order to test the enantioselectivity of cobalamin(I) towards racemic alkylating agents (Section 3. 3). The epoxides were made by the standard method of epoxidation of olefins with *m*-chloroperbenzoic acid[140].

Pure enantioners of (*R*)- and (*S*)-methyloxirane were kindly supplied by M.K. Ellis[141], as were mixtures of (*S*)-[2,2- $^{2}H_{2}$]-methyloxirane and (*R*)-methyloxirane, and of (*S*)-[2,2- $^{2}H_{2}$]-methyloxirane and (*S*)-methyloxirane, for use in the enantioselectivity studies.

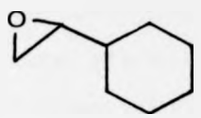
An attempt was made to prepare the enantiomers of 3,3-dimethyl-1,2-epoxybutane (46) (t-butyloxirane) *via* the diastereomeric acetal of 3,3-dimethylpropane-1,2-diol with (*S*)-camphorquinone[141]. Although the mixture of acetals was prepared, it could not be separated by fractional crystallisation.

2.6 [1,1- $^{2}H_{2}$]-ethanol *O*-tosylate

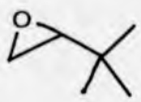
This compound was made by the reduction of acetyl chloride with lithium aluminium deuteride to give [1,1- $^{2}H_{2}$]-ethanol, by a similar method to that used by Murray and Williams[142]. ^{1}H NMR analysis (400 MHz, $D_{2}O$) showed no detectable unlabelled ethanol. In $D_{2}O$, the spectrum showed one signal, a (1 : 2 : 3 : 2 : 1) quintet at δ 1.08 J = 1.0 Hz, signifying a splitting by two equivalent deuterium atoms.

Tosylation of the labelled ethanol was accomplished by standard methods[56], and [1,1- $^{2}H_{2}$]-ethanol *O*-tosylate was isolated in 64 % yield. The ^{1}H NMR spectrum gave, as well as the signals for the tosyl group, a signal at δ 1.28 corresponding to three protons, a quintet (1 : 2 : 3 : 2 : 1) J = 1.0 Hz, showing that the methyl group was split by two equivalent deuterium atoms. Again, no unlabelled or singly labelled ethanol *O*-tosylate was detected, showing

that < 2 % of these species are present. Mass spectral analysis gave an M^+ ion at m/z 202, with < 2 % of the corresponding ions at m/z 200 or 201. The accurate mass (202.0626) was in good agreement with the calculated value ($C_9H_{10}D_2O_3S$, 202.0633).



(45)



(46)

2.7 EXPERIMENTAL SECTION

The reagents and solvents used in this work, were in general purified according to the methods in ref.[143]. Solvents were redistilled before use from an appropriate drying agent, and stored in tightly closed bottles, over drying agents when necessary.

2.7.1 Chromatography

i) For analytical thin-layer chromatography, commercial aluminium-foil-backed plates (silica gel 60, F_{254}, Merck Art. 5554) were used. Visualisation was with iodine, under UV light, or with $KMnO_4$ spray.

ii) When column chromatography was used to purify organic products, the method of Still *et al.*[144] (flash column chromatography) was employed. The columns were poured with dry silica gel (70 - 230 mesh, Merck Art. 7734) and the air forced out by the passage of the eluting solvent under pressure.

Eluting solvents were freshly made up, and where ratios of solvents are quoted, they refer to volume : volume.

iii) High pressure liquid chromatography: the instrument used was a Gilson model 303, with UV detection, the analytical column was Partisil PXS 1025, and the preparative column was Techoprep 5/20, for the separation of iodoalkanes. The eluent was hexane : triethylamine (99 : 1). I am grateful to Mr. J. Dennis for his help in optimising this separation.

2.7.2 Instrumentation

1) ^{1}H NMR spectra were recorded on a 60 MHz Hitachi Perkin-Elmer (model R24B), a 220 MHz Perkin-Elmer (model R34), a 300 MHz Bruker (model WH300) or a 400 MHz Bruker (model WH400) instrument. The instrument is specified by the quotation of its operating frequency. The ^{1}H NMR spectra are described in the experimental section by the chemical shifts, δ, in ppm, of the individual resonances. The chemical shifts are relative to the internal standard tetramethylsilane (TMS) (or 3-(trimethylsilyl)propionic acid (TSS) or 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) if the solvent was D_2O). Information about the nature of the peak is contained in brackets after the chemical shift. Firstly, the number of protons it corresponds to; secondly, the form of the peak: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = double doublet. J refers to the coupling constant in Hertz. A subscript (*e.g.* J_{gem}) refers to the nature of the coupling constant.

^{13}C NMR spectra were recorded at 22.63 MHz on a 90 MHz Bruker (model WH90) instrument. All the peaks were proton broad-band decoupled. The chemical shifts were measured relative to TMS, dioxan, or $CDCl_3$ as internal standards.

2) Infrared (ν) spectra were recorded on a Perkin-Elmer (model 257) instrument. The samples were run as liquid films on NaCl plates. The spectra are described by the wavenumber (cm^{-1}) of the peaks, which may be designated s = strong, m = medium, w = weak or br = broad.

3) Ultraviolet-visible (λ_{max}) spectra were recorded on a Shimadzu (model UV-365) or a Unicam (model SP800) instrument. The peaks are recorded in nanometers (nm) followed by the extinction coefficient in brackets, where applicable.

4) Mass spectra were recorded on a AEI MS-9 instrument, which gave electron impact mass spectra. The peaks are described as m/z, and the ion giving rise to the peak is shown in brackets. Metastable peaks (m*) are quoted as m/z, and the process which gives rise to the peak is shown in brackets e.g. (183 → 127).

5) Optical rotations were measured on a NPL Automatic Polarimeter (Type 243), in a 0.1 dm cell. The concentration, c, is given in g/ml, and the solvent is quoted.

6) The pH values of solutions were measured on a Corning PTI-5 Digital pH Meter, using a single glass electrode. For solution in $^{2}H_{2}O$, $p^{2}H = pH + 0.4$.

2.8 Synthetic methods

2.8.i) (*S*)-1,2-*O*-Isopropylideneglycerol (16)

Sodium periodate (8.0 g, 37 mmol) was dissolved in water (100 ml) and cooled in ice. 1,2;5,6-Di-*O*-isopropylidene-D-mannitol (6.84 g, 26 mmol) was added, and the mixture stirred for 1 h at 0 °. Ethanol (200 ml) was added, and a white precipitate of sodium iodate was seen. Sodium borohydride (2.0 g, 53 mmol) was added slowly, and the mixture stirred at 0 ° for 30 min, then at room temperature for a further 2 h. The ethanol was removed by rotary evaporation, the residue was filtered, saturated with NaCl, and extracted with ethyl acetate (5 x 30 ml). The combined organic extracts were washed with brine (30 ml), and $NaHCO_3$ (sat.) (30 ml), dried over Na_2SO_4 and the solvent evaporated. The colourless liquid (6.78 g, 98 %) was distilled (65 - 75 °, 12 mmHg) to yield (*S*)-1,2-*O*-isopropylideneglycerol ((*S*)- 16) (6.08 g, 88 %).

t.l.c. (silica gel, CH_2Cl_2) R_f = 0.4

^1H NMR (220 MHz, CCl_4) δ 1.30, 1.37 (2 x 3H, 2 x s, $(CH_3)_2C\overset{O}{\underset{O}{<}}$), 2.79 (1H, br s, -OH), 3.54 (2H, m, $-CH_2OH$), 3.70 (1H, dd, J = J = 7 Hz), 3.95 (1H, dd, J = J = 7 Hz, $-CH_2O-$), 4.12 (1H, m, $-\overset{|}{C}H-O-$).

Addition of D_2O removed the signal at δ 2.79, and split the signal at 3.54 into the AB portion of an ABX system δ_A = 3.47, δ_B = 3.61, J_{AB} = 15, J_{AX} = 6, J_{BX} = 5 Hz.

IR ν 3440 (br s), 2880 - 3000, 1385, 1375, 1060 cm^{-1}

Optical activity $[\alpha]_D$ = + 11.9 ± 1 ° (c = 0.401, MeOH)

$[\alpha]_D$ = + 10.4 ± 1 ° (c = 0.041, MeOH)

lit.[112] $[\alpha]_D$ = - 10.8 ° (c = 0.17, MeOH) for the opposite isomer

ii) (*R*)-1,2-*O*-Isopropylideneglycerol 3-*O*-tosylate ((*R*)-19)

((*S*)-16) (3.0 g, 23 mmol) was dissolved in dry pyridine (10 ml) and cooled to -10 ° in an ice-salt mixture. Tosyl chloride (5.0 g, 26 mmol) was dissolved in dry pyridine (10 ml) and added to the alcohol solution over 10 min, protected from moisture. The solution was stirred at -5 ° for 1 h, then left at 4 ° overnight. A white precipitate of pyridine hydrochloride formed.

The mixture was poured into ice-cold hydrochloric acid (2 M, 25 ml) and extracted with dichloromethane (5 x 5 ml). The combined organic fractions were washed with cold hydrochloric acid (2 M, 10 ml portions, until no pyridine remained), $NaHCO_3$ (sat.; 10 ml) and NaCl (sat.; 10 ml), dried with Na_2SO_4, and the solvent was evaporated to yield ((*R*)-19) (5.16 g, 79 %).

t.l.c. (silica gel, CH_2Cl_2) R_f = 0.7

^1H NMR	(220 MHz, CCl_4) δ 1.25, 1.28 (2 x 3H, 2 x s, $(CH_3)_2C$) 2.45 (3H, s, $ArCH_3$), 3.70 - 4.00 (4H, m, 2 x $-CH_2-O-$), 4.19 (1H, m, $-CH-O-$), 7.30 (2H, d, J = 9 Hz), 7.72 (2H, d, J = 9 Hz, ArH).
$\{^1H\}$ ^{13}C NMR	(22.63 MHz, $CDCl_3$) δ 21.4, 24.9, 26.3 (3 x CH_3) 69.3, 70.5, 72.7 ($HC_2-CH-CH_2$), 109.8 ($\underline{C}Me_2$), 127.8, 129.8, 145.0 (Ar).
Optical activity	$[\alpha]_D$ = -4.8 ± 0.5 ° (c = 0.40, EtOH), lit[13] -4.5 ° (c = 1, EtOH).

iii) (*S*) and (*R*)-Glycerol 1-*O*-tosylate ((*R*) and (*S*)-47)

Acetyl chloride (0.5 ml) was added to cold dry methanol (10 ml), to give a 3 % solution of HCl. ((*R* or *S*)-19) (0.3 g, 7 mmol) was added, and left 1 h at room temperature. Testing by t.l.c. showed that the acetal had reacted so the solution was evaporated to dryness. Residual HCl was removed by the addition of water (10 ml) and freeze-drying. The tosylate ((*R*) or (*S*)) (0.26 g, quantitative) was used

without further purification.

t.l.c. (silica gel, CH_2Cl_2 : MeOH (4 ; 1)) R_f = 0.2

^1H NMR (220 MHz, D_2O) δ 2.35 (3H, s, $ArCH_3$), 3.42 (2H, m, $-CH_2O-S$), 3.7 - 4.1 (3H, m, $-CH(OD)CH_2OD$), 7.40 (2H, d, J = 8 Hz), 7.74 (2H, d, J = 8 Hz, Ar-H).

iv) (*R*)-1,2-*O*-Isopropylideneglycerol ((*R*)-16)

1,2-*O*-Isopropylidene-L-ascorbic acid (1.13 g, 5.2 mmol) was dissolved in absolute ethanol (100 ml) and added over 1 h, to a stirred solution of sodium borohydride (0.2 g, 5.3 mmol) in ethanol (15 ml) at room temperature. The mixture was stirred for a further 4 h at room temperature, then was made alkaline by the addition of some NaOH pellets and NaOH (28 ml, 0.5 M). The mixture was stirred overnight and exactly neutralised with HCl (conc.). The solvents were removed under reduced pressure, with the addition of a little absolute ethanol towards the end of the evaporation to azeotrope remaining water, until the product remained as a dry white powder.

The powder was mixed with ethyl acetate (55 ml) and cooled to 0 °, and lead tetraacetate (10 g, 0.2 mol) was added in one portion. The yellow slurry was stirred for 1½ h at 0 °, then at 1½ h at room temperature. It was cooled again to 0 ° and filtered through celite into a cooled flask, to remove the precipitated lead diacetate, which was washed with cold ethyl acetate. The orange solution containing (*S*)-1,2-*O*-isopropylideneglyceraldehyde was added over 20 min to a solution of sodium borohydride (1.98 g, 52 mmol) in ethanol (40 ml) at 0 °. The black reaction mixture was stirred at 0 ° for 30 min and at room temperature for a further 2 h. It was made basic by adding NaOH pellets (2 g) and NaOH (0.5 M, 55 ml) and left overnight at 4 °.

Ether (30 ml) was added, the layers were separated and the aqueous phase was extracted with ether (3 x 15 ml). The combined organic phases were washed with brine (15 ml), were dried (Na_2SO_4), and the solvent was evaporated until ∿ 5 ml remained. Ether (15 ml) was added, the aqueous phase was saturated with NaCl, the layers were separated, and the aqueous phase was extracted with ether (4 x 15 ml). The combined organic phases were dried and evaporated as before. Yield of (*R*)-1,2-*O*-isopropylideneglycerol 0.33 g (48 %).

t.l.c. (silica gel, CH_2Cl_2) R_f = 0.4

^{1}H NMR	(220 MHz, CCl_4) δ 1.29, 1.35 (2 x 3H, 2 x s, $(CH_3)_2C$), 1.95 (1H, s, OH), 3.45, 3.59 (2H, AB portion of ABX, J_{AB} = 15, J_{AC} = 6, J_{BX} = 5 Hz, $-CH_2OH$), 3.70 (1H, dd, J = J = 7 Hz), 3.93 (1H, dd, J = J = 7 Hz, $-CH_2-O-$), 4.09 (1H, m, $-\overset{|}{C}H-O-$).
IR	3440, 2880 - 2950, 1385, 1375, 1060 cm^{-1}
Optical activity	$[\alpha]_D$ = -10.9 ± 1 ° (c = 0.10, MeOH) lit.[112] $[\alpha]_D$ = -10.8 ° (c = 0.17, MeOH).

v) (*S*)-2.3-*O*-Isopropylideneglycerol 1-*O*-tosylate ((*S*)-19)

This compound was prepared from (*R*)-1,2-*O*-isopropylideneglycerol (300 mg, 2.3 mmol) by the method in (ii) using tosyl chloride (0.5 g, 2.6 mmol) in dry pyridine (3 ml).

Yield 0.42 g, 65 %.

t.l.c. (silica gel, CH_2Cl_2) R_f = 0.7

^{1}H NMR	(220 MHz, CCl_4) δ 1.25, 1.27 (2 x 3H, 2 x s, $(CH_3)_2C$), 2.45 (3H, s, $ArCH_3$), 3.68 - 4.03 (4H, m, 2 x $-CH_2-O-$), 4.18 (1H, m, $-\overset{|}{C}H-O-$), 7.30 (2H, d, J = 9 Hz), 7.75 (2H, d, J = 9 Hz).

vi)a (*RS*)-4-(2-Hydroxyethyl)-2,2-dimethyl-1,3-dioxolan (20)

(*RS*)-Butane-1,2,4-triol (1 g, 9.4 mmol) was dissolved in dry acetone (50 ml) and tosic acid (25 mg). The solution was stirred at room temperature overnight. The solvent was evaporated under reduced pressure, and the residue was taken up in ether, washed with Na_2CO_3 (sat., 2 x 20 ml) and dried (Na_2CO_3) and the solvent was evaporated. The oil (20) was distilled (Kugelrohr, 110 °, 11 mmHg) Yield 1.07 g, 78 %.

t.l.c. (silica gel, CH_2Cl_2 : MeOH (9 : 1) R_f = 0.6

^1H NMR (220 MHz, CCl_4) δ 1.29, 1.35 (2 x 3H, 2 x s, $(CH_3)_2C$), 1.71 (2H, m, C-CH_2-C), 2.35 (1H, t, J = 5 Hz, -OH), 3.47 (1H, dd, J = 8, 8 Hz, HC<u>H</u>-O-), 3.55 (2H, m, -CH_2OH), 3.97 (1H, dd, J = 8, 6 Hz, <u>H</u>CH-O-), 4.15 (1H, m, -C<u>H</u>-O-)

$\{^1H\}$ ^{13}C NMR (22.63 MHz, CCl_4), δ 25.4, 26.6 ($(\underline{C}H_3)_2C$), 35.9 (-CH_2-), 59.2 (-CH_2OH), 69.6 (-CH_2-O-), 74.5 (-CH-O-), 110.2 ($\underline{C}Me_2$).

vi)b (*RS*) and (*S*)-4-(2-Hydroxyethyl)-2,2-dimethyl-1,3-dioxolan 2-*O*-tosylate ((*RS*) and (*S*)- 21)

(*RS*) and (*S*)-4-(2-Hydroxyethyl)-2,2-dimethyl-1,3-dioxolan (20) (0.74 g, 5.3 mmol) were tosylated by the same method[56] as in (ii) using tosyl chloride (1.15 g, 6 mmol) in pyridine (6.5 ml) at - 5 °. Yield (1.2 g, 74 %) ((*S*)- 21); (1.23 g, 81 %) ((*RS*)- 21)

t.l.c. (silica gel, CH_2Cl_2) R_f = 0.75

^1H NMR (220 MHz, CCl_4) δ 1.22, 1.27 (2 x 3H, 2 x s, $(CH_3)_2C$), 1.8 (2H, m, C-CH_2-C), 2.45 (3H, s, Ar-CH_3), 3.43 (1H, dd, J = 8, 8 Hz, HC<u>H</u>-O-), 3.95 (1H, dd, J = 8, 7 Hz, <u>H</u>CH-O), 4.06 (3H, m, -C<u>H</u>-O-, -CH_2OTs), 7.30 (2H, d, J = 9 Hz), 7.74 (2H, d, J = 9 Hz, Ar-H).

vii) (*S*)-γ-Carboxyl-γ-butyryl lactone ((*S*)- 22)

L-Glutamic acid (9.0 g, 61 mmol) in hydrochloric acid (10 %, 50 ml) was cooled to 0 °, and a solution of $NaNO_2$ (6.3 g, 92 mmol) in water (15 ml) was added over 4 h, keeping the temperature below 5 °. It was then stirred at room temperature overnight. The water was evaporated under reduced pressure, and a pale yellow oil and some insoluble material were obtained. Ethyl acetate (120 ml) was added, and the solution was filtered, dried (Na_2SO_4), and the solvent was evaporated to give ((*S*)- 22) as a yellow oil (7.5 g, 94 %).

^1H NMR (220 MHz, D_2O) δ 2.3 - 2.8 (4H, m, $-CH_2-CH_2-$) 5.2 (1H, dd, J = 5, J = 8 Hz, $-O-\overset{|}{C}H-CO_2D$)

Optical activity $[\alpha]_D$ = + 16.1 ± 1 ° (c = 0.02, EtOH)

lit.[115], $[\alpha]_D$ = + 15.6 ° (c = 0.02, EtOH)

viii) (*S*)-Pentane-1,2,5-triol ((*S*)-22a)

((*S*)- 22) (4 g, 31 mmol) was dissolved in dry THF (10 ml). Lithium aluminium hydride (2.0 g, 53 mmol) was placed in a dry 3-necked flask, which was fitted with a condenser and dropping funnel and flushed with dry nitrogen, and THF (75 ml) was added. The lactone solution was added over 15 min from the dropping funnel, and the stirred solution was refluxed overnight.

After cooling, water (7 ml) was added cautiously, and the suspension stirred for 30 min, then filtered. The solid was extracted with ethanol for 4 h, using a Soxhlet apparatus. The ethanolic extract was combined with the THF filtrate, and the solvents were evaporated. The resulting yellow oil was dissolved in water (10 ml), and the solution exactly neutralised with 50 % aqueous sulphuric acid. The water was evaporated to give ((*S*)-22a) 3.5 g (95 % but probably contaminated with water).

Kugelrohr distillation (140 - 150 °, 0.1 - 0.2 mmHg) gave 2.6 g (51 %) of ((*S*)-22a)

t.l.c. (silica gel, CH_2Cl_2 : MeOH (3 : 1)) R_f = 0.1

^1H NMR (220 MHz, D_2O) δ 1.3 - 1.8 (4H, m, $-CH_2-CH_2-$), 3.4 - 3.8 (5H, m, $-CH_2-OD$, $-CH(OD)CH_2OD$)

IR ν 3310 (br), 2860 - 2940, 1430, 1100 cm^{-1}

$[\alpha]^D$ = - 12.1 ± 1 ° (c = 0.04, EtOH), lit[115] -11.6 ° (c = 0.04, EtOH)

ix) (*S*)-4-(3-Hydroxypropyl)-2,2-dimethyl-1,3-dioxolan ((*S*)-23)

((*S*)-22a) (0.8 g, 6.7 mmol) was dissolved in dry acetone (25 ml) and tosic acid (25 mg) was added. The flask was stoppered and left overnight. The solvent was then evaporated and the residue was taken up in ether, was washed with $NaHCO_3$ (sat., 2 x 20 ml), dried (Na_2CO_3) and the solvent was evaporated. Yield of ((*S*)-23) (0.5 g, 51 %). Unreacted triol ((*S*)-22a) was recovered by evaporation of the sodium bicarbonate solution to leave a white slurry. The residue was taken up,in ethanol, and was filtered. The solid was washed with additional ethanol, and the combined ethanolic extracts were dried (Na_2CO_3) and the solvent was evaporated. The triol was taken up in acetone (25 ml) and tosic acid was added (25 mg), and the solution left for 2 days. Work-up as above gave ((*S*)-23) (0.27 g, 27 %). Total yield (0.77 g, 78 %).

t.l.c. (silica gel, CH_2Cl_2) R_f = 0.4.

^1H NMR (220 MHz, CCl_4) δ 1.28, 1.34 (2 x 3H, 2 x s, $(CH_3)_2C$), 1.4 - 1.5 (4H, m, $-CH_2-CH_2-$), 2.2 (1H, br s, OH), 3.41 (1H, dd, J = 7, 7 Hz, $-HCH-O-$), 3.57 (2H, m, $-CH_2OH$), 3.95 (1H, dd, J = 7, 7 Hz, $-HCH-O-$), 4.01 (1H, m, $-CH-O$).

$\{^1H\}$ ^{13}C NMR (22.63 MHz, CCl_4) δ 25.3, 26.5 ($(\underline{C}H_3)_2C$), 28.3, 29.8 ($-(CH_2)_2-$), 62.2 ($-CH_2OH$), 67.4 ($-CH_2-O-$), 76.8 ($-\dot{C}H-O-$), 110.0 ($\underline{C}Me_2$).

x) (*S*)-4-(3-Hydroxypropyl)-2,2-dimethyl-1,3-dioxolan 3-*O*-tosylate

((*S*)- 23) (0.27 g, 17 mmol) was tosylated by the method in (ii), to yield ((*S*)- 24) (0.51 g, 96 %).

t.l.c. (silica gel, CH_2Cl_2 : MeOH (10 : 1)) R_f = 0.7

^1H NMR (220 MHz, CCl_4) δ 1.23, 1.27 (2 x 3H, 2 x s, $(CH_3)_2C$), 1.4 - 1.9 (4H, m, $-CH_2-CH_2-$), 3.3 - 3.4 (1H, m, $-\dot{C}H-O-$), 3.85 - 4.10 (4H, m, $-CH_2-O-$), 7.28 (2H, d, J = 8 Hz), 7.73 (2H, d, J = 8 Hz, Ar-H).

xi) 4-Hydroxy-10,10-dimethyl-9,11-dioxabicyclo[6.3.0]undecane (25)

This compound was synthesised by the method of Yates *et al.*[123] and Golding *et al.*[56] (Scheme 2.4) from cycloocta-1,4-diene. A mixture of diastereoisomers was obtained.

^1H NMR (220 MHz, CCl_4) δ 1.25 (6H, s, $(CH_3)_2C$), 1.3 - 2.2 (10H, m, $-CH_2-$), 3.5 - 3.8 (3H, m, $-\dot{C}H-O-$).

xii) 4-Hydroxy-10,10-dimethyl-9,11-dioxabicyclo[6.3.0]undecane 4-*O*-tosylate (27)

The above compound was prepared by the tosylation of (25) by standard methods[56] in 90 % yield.

^1H NMR (220 MHz, CCl_4) δ 1.25 (6H, s, $(CH_3)_2C$), 1.25 - 2.25 (10H, m, $-CH_2-$), 2.45 (3H, s, $ArCH_3$), 3.5 - 3.8 (2H, m, $-\dot{C}H-O-C$), 4.40, 4.60 (1H, 2 x m, $-\dot{C}H-O-S$), 7.28 (2H, d, J = 8 Hz), 7.71 (2H, d, J = 8 Hz, ArH).

The diastereoisomers of (27) are only distinguished by the signal of C4-H, which gives signals at δ 4.40 and 4.60 for the two diastereoisomers (27 a) and (27 b).

Separation of the diastereoisomers: the oil containing the mixture of (27 a) and (27 b) was mixed with an equal volume of light petrol (40 - 50 °) and cooled in ice. A white crystalline solid separated, was collected by filtration and washed with a little cold light petrol. Recrystallisation at -20 ° from CH_2Cl_2 ; light petrol (1 : 10) gave white crystals (14 %), shown by ^1H NMR spectroscopy to be largely pure (27 a).

The combined filtrates and mother liquors were evaporated and were found to contain (27 a) and (27 b) in the ratio (1 : 4) with a small amount of the elimination product (26).

10,10-Dimethyl-9,11-dioxabicyclo[6.3.0]undec-4-ene (26)

^1H NMR (220 MHz, CCl_4) δ 1.25 (6H, s, $(CH_3)_2C$), 1.25 - 1.5 (2H, m, $-CH_2-$), 1.95 - 2.3 (6H, m, $-CH_2-$), 3.7 - 3.8 (2H, m, (-CH-O-), 5.58 (2H, m, -CH = CH-).

xiii) n-Hexanol *O*-trifluoromethanesulphonate[117]

A solution of n-hexane-1-ol (0.71 g, 7 mmol) and pyridine (0.56 ml, 7 mmol) in dichloromethane (2 ml) was added over 45 min to a solution of trifluoromethanesulphonyl anhydride (2 g, 7 mmol) in dichloromethane (10 ml) at 0 °. The solution was left for a further 30 min at 0 °, during which time a white precipitate formed. The mixture was washed with water (2 x 10 ml), dried (Na_2SO_4) and the solvent evaporated.

Yield 1.34 g (82 %)

^1H NMR (220 MHz, CCl_4) δ 0.93 (3H, t, $-CH_3$), 1.3 - 1.6 (6H, m, $-(CH_2)_3-$), 1.75 - 1.95 (2H, m, $-CH_2-O-$), 4.5 (2H, t, $-CH_2-O-$).

xiv) Cyclooctanol *O*-trifluoromethanesulphonate

A solution of cyclooctanol (0.90 g, 7 mmol) and pyridine (0.56 ml, 7 mmol) in dichloromethane (2 ml) was added over 30 min to trifluoromethanesulphonyl anhydride (2 g, 7 mmol) in dicloromethane (10 ml), cooled to -15 °. It was left overnight at -20°, during which time a white precipitate formed, and the solution darkened to yellow. The solution was washed with ice-cold water (2 x 10 ml), dried ($MgSO_4$) and the solvent was evaporated. The ^1H NMR spectrum showed that cyclooctene was present as a major component, with triflate present in *ca.* 20 % yield).

^1H NMR (220 MHz, CCl_4) δ 1.2 - 2.2 (14H, m, $-(CH_2)-$), 4.55 (1H, m, -CH-OTf).

xv) 2,2-Dimethylpent-4-enal (31)[121]

Allyl alcohol (29.0 g, 0.5 mol) and 2-methylpropanal (54.0 g, 0.75 mol) in *p*-cymene (100 ml) with *p*-toluenesulphonic acid (0.125 g, 0.7 mmol) were heated under reflux with a 12 inch packed column below a Dean-Stark trap, fitted with a condenser and drying tube. The mixture was refluxed for 46 h, during which time, 9.4 ml of water separated. The bath temperature was raised from 105 ° to 150 ° during the course of the reaction, to maintain the reflux. The Dean-Stark trap was then replaced by a distillation head and a condenser, and the reaction mixture was fractionally distilled. 2,2-Dimethylpent-4-enal (b.p. 124 - 130°, 760 mmHg) was obtained (38.6 g, 69 %), with a further ∿ 7 g contaminated with *p*-cymene (total yield ∿ 80 %).

^1H NMR (60 MHz, CCl_4) δ 1.0 (6H, s, $(CH_3)_2C$), 1.15 (2H, d, J = 6 Hz, $-CH_2-$), 4.7 - 6.0 (3H, m, $-CH = CH_2$), 9.2 (1H, s, -CHO).

IR (liquid film) ν 1725, 1640 cm^{-1}.

xvi) 2,2-Dimethylpent-4-en-1-ol (32)

2,2-Dimethylpent-4-enal (11.2 g, 0.1 mol), methanol (60 ml) and water (40 ml) were cooled in ice, and sodium borohydride (1.4 g, 0.037 mol) in 0.2 M NaOH (20 ml) was added dropwise, keeping the temperature below 22 °. The mixture was stirred at 10 ° for 1 h.

Most of the methanol was removed by distillation through a short Vigreux column. The residue was diluted with water (60 ml) and extracted with dichloromethane. The organic extracts were washed with water and dried ($MgSO_4$). The dichloromethane was removed by flash distillation and the alcohol was distilled at 78 - 80 °C (40 - 44 mmHg) or 154 °C (760 mmHg). 8.03 g (71 %) of 2,2-dimethylpent-4-en-1-ol (32) was recovered.

t.l.c. (Silica gel, ether : light petrol (2 : 1)) R_f = 0.4.

^1H NMR (300 MHz, $CDCl_3$) δ 0.88 (6H, s, $(CH_3)_2C$, 1.92 (1H, br s, -OH), 2.02 (2H, ddd, J = 6.5, 1.0, 1.0 Hz $C-CH_2-C$), 3.32 (2H, s, $-CH_2O-$), 5.02 - 5.09 (2H, m, $CH_2 = C$) 5.85 (1H, m, -CH = C).

IR (liquid film) ν 3400(br s), 3080, 2850 - 2960 (s), 1640, 1365, 1035 (s), 910.

MS m/z 96 ($M^+ - H_2O$), 83 ($M^+ - CH_2OH$), 81 ($M^+ - H_2O - CH_2$), 73 ($(CH_3)_2\overset{+}{C}CH_2OH$), 55 ($CH_2 = C(CH_3)\overset{+}{C}H_2$). m* 68.3 (96 → 81), 41.4 (73 → 55), 36.4 (83 → 55)

Measured mass for ($M^+ - H_2O$) = 96.0935, calculated for C_7H_{12} = 96.0939.

xvii) 1-*O*-Benzyl-2,2-dimethylpent-4-en-1-ol (33)

2,2-Dimethylpent-4-en-1-ol (5.7 g, 50 mmol) in tetrahydrofuran (85 ml, distilled from $LiAlH_4$) was cooled in ice under dry nitrogen. Sodium hydride (1.21 g, 50.6 mmol, prepared from 50 % dispersion in oil by

washing with petroleum ether (40 - 60 °) and removal of solvent *in vacuo*) was added in portions with cooling. The mixture was stirred at room temperature for 2 h and at 50 ° for 1 h until hydrogen evolution ceased. Tetra-n-butylammonium bromide (180 mg, 0.5 mmol, 1 mol %) was added, followed by benzyl bromide (6.1 ml, 50.3 mmol). The suspension was stirred at room temperature for 12 h, then hydrolysed by the addition of potassium carbonate (sat., 50 ml). The aqueous layer was extracted with dichloromethane (3 x 25 ml), and the combined organic layers were washed with K_2CO_3 (sat., 2 x 25 ml), dried (K_2CO_3) and the solvent was evaporated. Distillation of the residue gave the benzyl ether (33) (8.15 g, 80 %, b.p. 124 - 128 °, 11 - 12 mmHg).

t.l.c. (Silica gel, ether : light petrol (2 : 1)) R_f = 0.63.

^1H NMR (300 MHz, $CDCl_3$) δ 0.90 (6H, s, $(CH_3)_2C$), 2.05 (2H, ddd, J = 7.5, 1.0, 1.0 Hz, C-CH_2-C), 3.13 (2H, s, -CH_2-O-), 4.50 (2H, s, -OCH_2Ph), 4.96 - 5.04 (2H, m, CH_2 = C<), 5.72 - 5.86 (1H, m, -CH =), 7.23 - 7.37 (5H, m, Ar-H).

IR (liquid film) ν 3040 (w), 3020 (w), 2850 - 2990 (s), 1640, 1590 (w), 1500, 1455, 1385, 1365, 1100 (s), 740, 700 cm^{-1}.

MS m/z 204 (M^+ - CH_3), 163 (M^+ - CH_2 = CH—CH_2·) 91 ($PhCH_2^+$)

Measured mass 204.1501, calculated for $C_{14}H_{20}O$ 204.1514.

xviii) 1-*O*-Benzyl-4,5-epoxy-2,2-dimethylpentan-1-ol (34)

The olefin (33) (1.0 g, 4.90 mmol) in chloroform (20 ml), was cooled in ice, and *m*-chloroperbenzoic acid (85 %, 1.09 g, 5.4 mmol) was added in portions. The mixture was stirred at room temperature and monitored by ^{1}H NMR. After 4 h the intensity of the decreasing olefin signal (*gem*-dimethyl, δ 0.9, 6H, s) equalled the increasing impurity peak at δ 1.1. The intensity of the corresponding peak from the desired product (δ 1.0), showed that, at this time, the epoxide comprised about 70 % of the total.

The mixture was washed with sodium metabisulphite (5 %, 10 ml), sodium bicarbonate (10 %, 2 x 10 ml), and brine (10 ml), was dried, and the solvent was evaporated. The product was purified by flash chromatography (silica gel, light petrol (40 - 60 °) : ethyl acetate : triethylamine (94 : 5 : 1)). The column gave unreacted olefin (33), benzyl *m*-chlorobenzoate (37), 1-*O*-benzyl-4,5-epoxy-2,2-dimethylpentane-1-ol (34) (0.59 g, 55 %) and *O*-benzyl-2-(hydroxymethyl)-4,4-dimethyl-tetrahydrofuran (36). These compounds had R_f values of 0.9, 0.6, 0.54 and 0.4 respectively (t.l.c., Silica gel, CH_2Cl_2) and were identified by ^{1}H NMR and m.s.

1-*O*-Benzyl-4,5-epoxy-2,2-dimethylpentane-1-ol (34)

t.l.c. (Silica gel, CH_2Cl_2) R_f = 0.6

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.02 (6H, s, $(CH_3)_2C$) 1.50, 1.55 (2H, AB portion of ABX, J_{AB} = 14.1, J_{AX} = 6.4, J_{BX} = 5.3 Hz, C-CH_2-C), 2.40 (1H, dd, J_{gem} = 5.1, J_{vic} = 2.7 Hz), 2.70 (1H, dd, J_{gem} = 5.1, J_{vic} = 5.2 Hz, CH_2 - C), 2.97 (1H, m, -CH—), 3.19, 3.25 (2H, AB system, J_{AB} = 8.8 Hz, -O-CH_2-C(Me)$_2$-), 4.51 (2H, s, -OCH_2Ar), 7.2 - 7.4 (5H, m, Ar-H).

IR (thin film) ν 2860 - 3020 (s), 1610 (w), 1590 (w), 1500 (m), 1385, 1370, 1260, 1100, 740, 700 cm^{-1}

MS 220 (M^+), 91 ($PhCH_2^+$)

Measured mass 220.1462, calculated for $C_{14}H_{20}O_6$ 220.1463.

xix) 1-*O*-Benzyl-2,2-dimethylpentane-1,4,5-triol (35)

The epoxide (34) (0.5 g, 2.27 mmol) was added to a solution of lithium hydroxide (2.0 g, 48 mmol) in water (4 ml) and dimethyl sulphoxide (12 ml) (DMSO) and heated under a reflux condenser at 110 - 130 ° for 6 h. The mixture was cooled in ice, tested by t.l.c., and neutralised with concentrated HCl. Most of the DMSO was pumped off (0.3 mmHg, ∿ 50 °), and water (18 ml) was added to the slurry, which was extracted with CH_2Cl_2 (50 ml total). The organic extracts were combined, dried ($MgSO_4$) and evaporated. The remaining DMSO was removed *in vacuo*, and the diol was chromatographed (silica gel, CH_2Cl_2, with a gradient of 0 - 10 % methanol). The diol (35) (370 mg, 60 %) was pure by ^1H NMR.

t.l.c. (Silica gel, CH_2Cl_2 : MeOH (9 : 1)) R_f = 0.3

^1H NMR (300 MHz, $CDCl_3$) δ 0.93 (3H, s, CH_3-C), 1.01 (3H, s, CH_3-C), 1.35 (1H, dd, J_{gem} = 14.8, J_{vic} = 1.3 Hz), 1.50 (1H, dd, J_{gem} = 14.8, J_{vic} = 9.7 Hz C-CH_2-C(Me)$_2$-), 2.1, 2.3 (2H, 2 x br s, 2 x OH), 3.26, 3.30 (2H, AB system, J_{AB} = 9.1 Hz, -CH_2-OBn), 3.41 (1H, dd, J_{gem} = 10.8, J_{vic} = 7.2 Hz), 3.52 (1H, dd, J_{gem} = 10.8, J_{vic} = 3.6 Hz, -CH_2-O-), 3.82 (1H, m, -CH-O-), 4.54 (1H, s, Ar-CH_2-), 7.33 (5H, m. Ar-H). The broad signals about 2.1 and 2.3 disappeared on shaking with D_2O.

MS 238 (M^+), 220 ($M^+ - H_2O$), 207, 187, 91 ($PhCH_2^+$)
Measured mass 238.1579, calculated for $C_{14}H_{22}O_3$ = 238.1569.

xx) *O*-Benzyl-4-(3-hydroxy-2,2-dimethylpropyl)-2,2-dimethyl-1,3-dioxolan (38)

The diol (35) (0.5 g, 2.1 mmol) was placed in a dry flask and acetone (2.5 ml, 34 mmol) was distilled from K_2CO_3 into the flask with exclusion of moisture. Copper(II) sulphate (anhydrous; 350 mg, 2.2 mmol) and sulphuric acid (conc.; 20 µl, ∿ 0.5 mmol) were added. The flask was stoppered and sealed, and left overnight. The reaction mixture was neutralised and dried by the addition of K_2CO_3 (anhydrous). After filtration and evaporation, the crude acetonide (0.48 g, 82 %) was chromatographed (silica gel, 5 % ethyl acetate, 1 % triethylamine, 94 % light petrol (40 - 60 °)). The pure acetonide (38) was obtained (0.41 g, 70 %)

t.l.c. (silica gel, CH_2Cl_2) R_f = 0.6

^{1}H NMR (300 MHz, $CDCl_3$) δ 0.96 (3H, s), 0.97 (3H, s, $(CH_3)_2C$), 1.33 (3H, s), 1.38 (3H, s, $(CH_3)_2C\langle^{O}_{O}$) 1.55 (1H, dd, J_{gem} = 14.1, J_{vic} = 5.6 Hz), 1.67 (1H, dd, J_{gem} = 14.1, J_{vic} = 6.5 Hz, C-CH_2-C), 3.15, 3.18 (2H, AB system, J_{AB} = 8.8 Hz, C-CH_2-O), 3.43 (1H, dd, $J_{gem} = J_{vic}$ = 7.8 Hz), 4.02 (1H, dd, J_{gem} = 7.8, J_{vic} = 5.8 Hz, -H_2C-O), 4.16 (1H, m, -CH-O), 4.50 (2H, s, -CH_2-Ar), 7.26 (5H, m, Ar-H).

MS m/z 278 (M^+), 263 ($M^+ - CH_3$), 220 ($M^+ - (CH_3)_2CO$), 202 (220 - H_2O), 187 ($M^+ - CH_3Ph$), 171 ($M^+ - CH_3 - CH_3Ph$), 91 ($PhCH_2^+$)

m* 186.5 (220 → 202), 174.1 (278 → 220),
111.2 (263 → 174.1).

xxi) 4-(3-Hydroxy-2,2-dimethylpropyl)-2,2-dimethyl-1,3-dioxolan (29)

The benzyl ether (38) (0.30 g, 1.08 mmol) was dissolved in absolute ethanol (21 ml) and cooled in ice. 10 % Pd/C (150 mg) was added, and the mixture was hydrogenated at room temperature and atmospheric pressure. Hydrogen (26.8 ml, 1.07 mmol) was taken up within 20 min, and then uptake ceased. The reaction mixture was filtered through celite, and the ethanol was evaporated. The alcohol (29) (160 mg, 79 %) was chromatographed (silica gel, light petrol : ethyl acetate : triethylamine (89 : 10 : 1)) to give the pure alcohol (29)(155 mg, 77 %). The alcohol was stored at -20 °, with triethylamine (∿ 5 %) added to prevent decomposition.

t.l.c. (silica gel, CH_2Cl_2) R_f = 0.3

^{1}H NMR (300 MHz, $CDCl_3$) δ 0.93, 0.94 (2 x 3H, 2 x s, $(CH_3)_2C$) 1.37, 1.41 (2 x 3H, 2 x s, $(CH_3)_2C<^{O-}_{O-}$), ∿ 1.4 (1H,), 1.62 (1H, dd, J_{gem} = 14.7, J_{vic} = 9.6 Hz, C-CH_2-C), 3.3 (1H, br s, -OH), 3.28, 3.40 (2H, AB system, J_{AB} = 11.4 Hz, -CH_2OH), 3.46 (1H, dd, $J_{gem} = J_{vic}$ = 8.0 Hz), 4.06 (1H, dd, J_{gem} = 8.0, J_{vic} = 5.9, -CH_2-O-), 4.19 (1H, m, -CH-O-).

IR ν 3440 (s), 2860 - 2990 (s), 1485, 1380, 1370, 1050.

MS 173 (M^+-CH_3), 158 (M^+-2(CH_3)), 131, 113
m* 97.5 (131 → 113)

xxii) 4-(3-Iodo-2,2-dimethylpropyl)-2,2-dimethyl-1,3-dioxolan (28)

First preparation: Triphenoxymethylphosphonium iodide (49)(1.35 g, 3 mmol), which was washed with ethyl acetate (2 x 3 ml), dry ether (3 x 3 ml) and dried in a stream of dry nitrogen and pumped at 0.5 mmHg before weighing, was dissolved in DMF (1.2 ml), benzene (1 ml) and pyridine (12 µl) in a dry, serum-capped flask. The alcohol (29) was added, and the mixture left under nitrogen, protected from moisture, for 2 h. ^{1}H NMR monitoring showed no further changes after 30 min. Methanol (0.25 ml) was added to decompose the remaining methiodide, and the mixture poured into NaOH (0.1 M, 10 ml) and light petrol (40-60 °)(10 ml), and shaken. The organic phase was separated and washed with NaOH (0.1 M, 2 x 10 ml) and water (2 x 5 ml), dried (K_2CO_3) and the solvent evaporated. The residue was free from phenol, but contained diphenyl methylphosphonate as well as the required iodide. Flash column chromatography (silica gel, light petrol (40 - 60 °) : ethyl acetate : triethylamine (89 : 10 : 1)) separated these. The iodide-containing fractions (62 mg) were found to contain a mixture of products, which were identified by m.s. and ^{1}H NMR spectroscopy to be 4-(3-iodo-2,2-dimethylpropyl)-2,2-dimethyl-1,3-dioxolan (28) and 2-iodomethyl-4,4-dimethyltetrahydrofuran (40) in the ratio (1 : 2).

t.l.c. (silica gel, light petrol (40 - 60 °) : ethylacetate (3 : 1)

R_f = 0.7

^{1}H NMR (300 MHz, $CDCl_3$) δ 1.08, 1.11 (2 x 3H, 2 x s, $(CH_3)_2C$) 1.35, 1.40 (2 x 3H, 2 x s, $(CH_3)_2C\langle^{O-}_{O-}$), ∿ 1.55 (1H, dd, J_{gem} = 14.4, J_{vic} = 3.9 Hz), 1.70 (1H, dd, J_{gem} = 14.4, J_{vic} = 8.0 Hz), ($-CH_2-C$), ∿ 3.25 (2H, AB system, J_{AB} masked, $-CH_2-I$), 3.46 (1H, dd, J_{vic} = J_{gem} = 7.7 Hz),

4.06 (1H, dd, J_{gem} = 7.6, J_{vic} = 5.9 Hz, O-H_2C-), ∿ 4.13 (1H, m, -CH-O-)

MS m/z 298 (M^+), 283 (M^+-CH_3), 268 (M^+-2 x CH_3) 223 (M^+-CH_3-CH_3CO_2H), 183 ($(CH_3)_2\overset{+}{C}CH_2I$), 171 ($M^+$-I), 128 ($HI^+$), 127 ($I^+$)

m* 175.7 (223 → 283)

Measured mass of M^+ = 298.0406, calculated for $C_{10}H_{19}O_2I$ = 298.0432.

2-Iodomethyl-4,4-dimethyltetrahydrofuran (40)

t.l.c. (silica gel, light petrol (40 - 60 °) : ethyl acetate (3 : 1))

R_f = 0.7

^1H NMR (300 MHz, $CDCl_3$) δ 1.10, 1.12 (2 x 3H, 2 x s, $(CH_3)_2C$) 1.45 (1H, dd, J_{gem} = 12.5, J_{vic} = 8.7 Hz), 1.94 (1H, dd, J_{gem} = 12.5, J_{vic} = 6.6 Hz, C-CH_2-C), 3.22, 3.28 (2H, AB portion of ABX system, J_{AB} = 9.9, J_{AX} = 5.4, J_{BX} = 6.6 Hz, -CH_2-O-), 3.55, 3.62 (2H, AB system, J_{AB} = 8.1 Hz, -CH_2I), ∿ 4.15 (1H, m, -HC-O-).

MS m/z 240 (M^+), 113 (M^+ - I)

Measured mass of (M^+ - I) = 113,0965, calculated for $C_7H_{13}O$ = 113.0966.

xxiii) 4-(3-Iodo-2,2-dimethylpropyl)-2,2-dimethyl-1,3-dioxolan (28)

Second preparation: The same method as that in the first preparation was used, with the addition of tetra-n-butylammonium iodide (1 g, 3.04 mmol). The alcohol (29)(100 mg, 0.53 mmol) was treated with triphenoxymethylphosphonium iodide (1.6 g, 3.54 mmol) in dry DMF (2.5 ml) and benzene (2.5 ml) with pyridine (10 µl).

Work-up was by the same method as in the first preparation, and the products were separated by flash column chromatography (silica gel, light petrol (40-60 °) : ethyl acetate : triethylamine (94 : 5 : 1)). The iodide-containing fractions (148 mg) again contained a mixture of products. An attempt was made to separate these by HPLC. An analytical column (partisil PXS 1025, hexane : triethylamine (99 : 1)) gave three peaks in ratio (6 : 2 : 1), and a preparative column (Techoprep 5/20, same eluent) gave two fractions (2 : 1). The ratios were calculated from the areas under the peaks, detected by UV 254 nm. The second fraction contained an equimolar mixture of the iodides produced in the first reaction. The first fraction contained a new product, tentatively identified by ^{1}H NMR and m.s. to be 4-iodomethyl-2,2,6,6-tetramethyl-1,3-dioxaheptane (41).

First HPLC fraction:

t.l.c. (silica gel, light petrol (40 - 60 °) : ethylacetate (3 : 1))

R_f = 0.7

^{1}H NMR (300 MHz, $CDCl_3$) δ 0.82, 1.03 (2 x 3H, 2 x s, $(CH_3)_2C$), 1.36, 1.41 (2 x 3H, 2 x s, $(CH_3)_2C\overset{O}{\underset{O}{<}}$), ∿ 1.4 (1H), 1.55 (1H, dd, J_{gem} = 13.6, J_{vic} = 1.3 Hz, C-CH_2-C), 3.05 (1H, dd, J_{gem} = 12.0, ^4J = 2.3 Hz, H$\underline{\overset{|}{C}}$H-O-), 3.11, 3.15 (2H, AB portion of ABX system, J_{AB} = 10.2, J_{AX} = 7.5, J_{BX} = 5.1 Hz, -CH_2I), 3.65 (1H, d, J_{gem} = 12.0 Hz, $\underline{H}\overset{|}{C}$H-O-), 3.95 (1H, m, -$\overset{|}{C}$H-O-)

MS m/z 298 (M^+), 283 (M^+-CH_3), 268 (M^+-2(CH_3)), 241, 223 (283 -CH_3CO_2H), 167 (M^+-CH_2I), 141 (CH_2I^+), 128 ($\overset{+}{H}$I), 127 (I^+).

xxiv) Triphenoxymethylphosphonium iodide (49)

Triphenyl phosphite (17 ml, 20.2 g, 64.9 mmol) and iodomethane (5.4 ml, 12.4 g, 87 mmol) were heated at reflux for 36 h, with protection from moisture (bath temperature 75 - 110 °). The reaction mixture was cooled and washed with ether. The yellow crystalline product was broken up, was ground in a mortar under dry ether, was washed with dry ether and stored under dry light petrol (40 - 60 °), in a stoppered flask. Yield ∿ 20 g, 70 %. The product was very hygroscopic, and turned dark brown on exposure to air, so was washed with ethyl acetate and ether until light yellow in colour before use, and was weighed in a stoppered flask under dry nitrogen.

^{1}H NMR (60 MHz, CCl_4) δ 3.02 (3H, d, J_{PH} = 16 Hz, P-CH_3), 7.2 (15H, br s, ArH).

xxv)a 4-Iodomethyl-2,2-dimethyl-1,3-dioxolan (30)

Model reaction for xxii)

Triphenoxymethylphosphonium iodide was placed in a weighed dry, 10 ml flask, was washed with ethyl acetate (3 x 3 ml), and ether (2 x 3 ml), a weighed serum cap was fitted, and the iodide was dried in a stream of dry nitrogen. The flask was reweighed, and the weight of the iodide was shown to be 1.6 g (3.54 mmol). Dry dimethylformamide (DMF) (1.7 ml), benzene (2 ml), and pyridine (10 µl) were added *via* the serum cap, and the phosphonium iodide was dissolved. 2,2-Dimethyl-4(hydroxymethyl)1,3-dioxolan (16) (150 µl, 160 mg, 1.2 mmol) was added, and the solution left for 2 h. ^{1}H NMR monitoring of a portion of the solution showed no changes after the first 5 min. Methanol (0.25 ml) was added to decompose the remaining phosphonium iodide. The reaction mixture was then diluted with pentane (5 ml) and poured into NaOH (0.1 M, 8 ml), and

shaken. The yellow iodine colour disappeared, and the phases were separated. The organic phase was washed with NaOH (0.1 M, 3 x 5 ml) until t.l.c. analysis showed that phenol was absent, then was washed once with water (5 ml), dried (K_2CO_3) and the solvent was evaporated. ^{1}H NMR analysis showed that the iodide (30) and diphenyl methylphosphonate (51) were present. Flash column chromatography (silica gel, light petrol (40 - 60 °) : ethyl acetate : triethylamine (89 : 10 : 1)) gave the pure iodide (30)(240 mg, 82 %).

4-Iodomethyl-2,2-dimethyl-1,3-dioxolan (30) : the properties of this compound were identical to those of the compound obtained by treating 4-hydroxymethyl-2,2-dimethyl-1,3-dioxolan-*O*-tosylate (19) with sodium iodide in refluxing acetone. B.p. 42 ° 12 mmHg.

t.l.c. (silica gel, CH_2Cl_2) R_f = 0.6

^{1}H NMR (60 MHz, CCl_4) δ 1.25, 1.35 (2 x 3H, 2 x s, $(CH_3)_2C<^{O-}_{O-}$) 3.0 - 3.15 (2H, m, $-CH_2I$), 3.5 - 3.45 (3H, m, $H_2C(O)-CH(O)-$)

IR ν 2880 - 2990, 1385, 1375, 1230, 1150, 1110, 840 cm^{-1}

Diphenyl methylphosphonate (51)

t.l.c. (silica gel, CH_2Cl_2) R_f = 0.2

^{1}H NMR (60 MHz, CCl_4) δ 1.84 (3H, d, J_{PH} = 18 Hz, $P-CH_3$) 7.2 (15H, br s, Ar-H)

xxv)b Neopentyl iodide (50) : Model reaction for xxii

Neopentyl iodide was prepared from neopentyl alcohol by the method of Landauer and Rydon[133] in 30 % yield. The alcohol (1.74 g, 20 mmol) was refluxed with triphenylphosphate (6.21 g, 20 mmol) and iodomethane (4.0 g, 28 mmol) for 60 h, and the iodide (50) was distilled out of the reaction mixture.

B.p. 61 - 62 °, 50 mmHg

^{1}H NMR (60 MHz, CCl_4) δ 1.05 (9H, s, $(CH_3)_3C$), 3.05 (2H, s, $-CH_2I$)

^{1}H NMR experiment : Neopentyl alcohol (88 mg, 1 mmol) and triphenoxymethylphosphonium iodide (1.36 g, 3 mmol, prepared as in xxv(a)), in DMF (1.3 ml) and benzene (2 ml), with pyridine (10 µl). A sample of the reaction mixture (0.75 ml) was transferred to a dry NMR tube, and spectra were recorded at intervals. At 30 °, the reaction was complete after 15 min, and neopentyl iodide (identical to that produced above) was isolated.

xxvi) 2,2,4-Trimethyl-1,3-dioxolan (52) : Model reaction for (xviii) to (xx).

Methyloxirane (1 ml, 0.83g, 14 mmol) was added to a solution of trifluoroboron etherate (50 µl) in acetone (9 ml). The mixture was left overnight protected from moisture. The resulting pale yellow solution was washed with K_2CO_3 (sat., 2 ml), dried (K_2CO_3), and fractionally distilled, to give (52) (0.8 g, 48 %), as a colourless liquid (b.p. 95 - 99 °, 760 mmHg).

Its properties were identical to those of the compound obtained by treating propane-1,2-diol with acetone in the presence of a catalytic amount of tosic acid.

^{1}H NMR (60 MHz, CCl_4) δ 1.2 (3H, d, $-CH_3$), 1.25, 1.3 (2 x 3H, 2 x s, $(CH_3)_2C\overset{O}{\underset{O}{<}}$), 3.1 - 3.4) (1H, m, $-\dot{C}HO-$) 3.7 - 4.2 (2H, m, $-CH_2-O-$)

MS m/z 116 (M^+), 101 (M^+-CH_3)

xxvii) Cyclohexyloxirane (45)

Vinylcyclohexane (1.10 g, 10 mmol) was dissolved in cold dichloromethane (25 ml) and cooled to -5 °. *m*-Chloroperbenzoic acid (85 %, 2.22 g, 11 mmol) was added in portions and the mixture left at -20 ° overnight. The ^{1}H NMR spectrum of the reaction mixture showed that very little starting material remained, so the mixture was filtered to remove precipitated *m*-chlorobenzoic acid. The solution was washed with sodium bisulphite (2 %, 10 ml) then sodium bicarbonate (10 %, 3 x 7 ml) and brine (10 ml). The organic solution was dried ($MgSO_4$) and the solvent was evaporated to give (45) (1.14 g) contaminated with minor amounts of vinylcyclohexane and *m*-chlorobenzoic acid. Kugelrohr distillation (∿ 90 °, 50 mmHg) gave (45)(0.94 g, 74 %)

^{1}H NMR (220 MHz, CCl_4) δ 1.0 - 1.25 (6H, m, -C$\overset{|}{H}_{ax}$-), 1.5 - 1.9 (5H, m, -C$\overset{|}{H}_{eq}$-), 2.35 (1H, m, -HC$\overset{|}{H}$-O) 2.54 (2H, m, $-CH_2O-$)

xxviii) 1,2-Epoxy-3,3-dimethylbutane (t-butyloxirane)(46)

This compound was made in the same way as cyclohexyloxirane (45), using *m*-chloroperbenzoic acid (6.07 g, 30 mmol), 85 %) in dichloromethane (90 ml) to epoxidise 3,3-dimethylbut-1-ene (3.8 ml, 2.5 g, 30 mmol) ^{1}H NMR monitoring showed some olefin remaining after 3 days at -20 °, but the reaction was stopped, and the t-butyloxirane isolated in the usual way. Distillation of the dichloromethane solution of the oxirane led to isolation of the product (b.p. 86 °, 760 mmHg), contaminated with dichloromethane (∿ 55 % yield of t-butyloxirane). A portion of the solution was purified by preparative GC (SE 30, Porapak 100 mm column, 100 °) to give pure t-butyloxirane (0.27 g).

^{1}H NMR (220 MHz, CCl_4) δ 0.90 (9H, s, $(CH_3)_3C$)
2.46 (2H, AB portion of ABX, J_{AB} = 5 Hz, $-CH_2-O-$)
2.57 (1H, X portion of ABX, J = 3, 4 Hz, $-\overset{1}{C}H-O$)

xxix) 3,3-Dimethylbutane-1,2-diol (53)

3,3-Dimethylbut-1-ene (5 ml, 3.27 g, 39 mmol) was cooled to 0 °, in a flask equipped with a thermometer, stirrer bar and dropping funnnel, with Teflon collars on the ground glass joints. A solution of hydrogen peroxide (30 %, 11 ml, 100 mmol) in cold formic acid (50 ml) was added dropwise, at a rate to keep the temperature below 10 °. The reaction mixture was then stirred without cooling for 15 min, and then heated at 35 ° for 3 h. ^{1}H NMR showed that no olefin remained. The solvent was evaporated at reduced pressure. A test for hydrogen peroxide (starch-iodide paper) was negative. The residue was cooled and KOH (10 g, 0.18 mol) in methanol (50 ml) was added cautiously, and stirred overnight at 30 °. The methanol was evaporated and water (50 ml) was added. The solution was neutralised and continuously extracted for 48 h with ether. The ethereal extract was dried ($MgSO_4$) and evaporated to give 3,3-dimethylbutane-1,2-diol (53) (2.3 g, 50 %). Kugelrohr distillation (108 °, 15 mmHg) gave 2.23 g (48 %) of (53) as a colourless oil which crystallised on cooling.

t.l.c. (silica gel, CH_2Cl_2) R_f = 0.1

^{1}H NMR (220 MHz, $CDCl_3$) δ 0.92 (s, 9H, $(CH_3)_3C$)
3.07 (2H, s, -OH), 3.39 (1H, dd, J = 2, 9.5 Hz)
3.50 (1H, dd, J = 9.5, 10.5 Hz), 3.75 (1H, dd,
J = 10.5, 2 Hz $-CH(OD)-CH_2OD$).

xxx) *Attempted resolution 141 of the enantiomers of 3,3-dimethylbutane-1, 2-diol via the diastereoisomeric acetals of camphorquinone*

Camphorquinone (0.61 g, 3.7 mmol) and tosic acid (60 mg, 0.35 mmol) were dissolved in benzene (50 ml) and 3,3-dimethylbutane-1-2-diol (1.3 g, 11 mmol) was added. The mixture was refluxed, using a Soxhlet apparatus containing oven-dried silica gel to remove water produced in the reaction. Samples were evaporated at intervals and the ^{1}H NMR spectra were recorded. After 24 h the spectra were no longer changing, so the benzene was evaporated and ether (25 ml) was added. The ethereal solution was washed with water (2 x 10 ml) and dried (Na_2CO_3) and the ether was evaporated. The mixture of acetals was purified by column chromatography (silica gel, light petrol (40 - 60 °) : ethyl acetate (7 : 1)), the acetals being eluted before unreacted camphorquinone or the diol (53). The R_f values of these compounds (silica gel, same solvent) were 0.6, 0.4 and 0.1, respectively. The acetals were obtained in 0.6 g (63 %) yield. ^{1}H NMR analysis showed a mixture of isomers which could not be separated by crystallisation from light petrol (40 - 60 °) or from aqueous methanol. Attempts to separate them using HPLC were also not successful.

t.l.c. (silica gel, light petrol (40 - 60 °) : ethyl acetate : (7 : 1)) R_f = 0.6

^{1}H NMR (220 MHz, CCl_4) δ 0.85 - 1.0 (18H, m, $-CH_3$),
1.0 - 2.1 (5H, m, $-CH_2-CH_2-\dot{C}H-$)
3.4 - 4.0 (3H, m, $-OCH_2-\dot{C}H-O$)

xxxi) [1,1 $^{2}H_2$]-ethanol

This compound was made in a way analogous to that used by Murray and Williams[142] to prepare ^{14}C-labelled ethanol.

Lithium aluminium deuteride (0.56 g, 13.3 mmol) and diglyme (11 ml) were placed in a dried, two-necked flask equipped with a stirrer bar, dry-ice-acetone condenser, nitrogen inlet and a flask containing acetyl chloride (1.0 ml, 13.3 mmol) attached to the side arm. The suspension was stirred under dry nitrogen to remove dissolved oxygen, and was cooled to 0°. Warming the acetyl chloride to ~ 60 ° caused it to distil slowly into the reaction flask. When the transfer was complete, the mixture was stirred for a further 2 h, at 0 °, then the remaining $LiAlD_4$ was decomposed by the dropwise addition of diethyleneglycol butyl ether (10 ml) (1-(2-(2-hydroxyethoxy)ethoxy)butane).

The labelled ethanol was distilled out of the reaction mixture by heating to 120 °, and trapping the ethanol in a clean side-arm flask cooled in dry ice-acetone. Yield 46 mg (7 %).

N.B. A trial experiment using $LiAlH_4$ on the same scale gave unlabelled ethanol (0.35 g, 53 %).

^{1}H NMR (400 MHz, D_2O) δ 1.08 (quintet 1 : 2 : 2 : 2 : 1, J = 1.05 Hz, $\underline{CH_3}$-CD_2OD).

xxxii) [1,1- $^{2}H_2$]-ethanol 1-*O*-tosylate (54)

[1,1- $^{2}H_2$]-ethanol (46 mg, 0.96 mmol) was cooled to -10 ° and added to a solution of tosyl chloride (0.27 g, 1.4 mmol) in dry pyridine (1.1 ml), also cooled to -10 °. The mixture was left at -20 ° for 3 h, protected from moisture, and a white precipitate of pyridine chloride formed. Hydrochloric acid (4 M; 4 ml) at -5 ° was added to the solution, which was extracted with dichloromethane

(5 x 1 ml), washed with cold HCl (2 M; 1 ml), dried ($MgSO_4$) and the solvent was evaporated. 124 mg of the crude tosylate was recovered, which was purified by preparative thin layer chromatography (silica gel, CH_2Cl_2), to remove a fast-running, unidentified impurity. The pure tosylate (90 mg, 46 %) contained < 2 % unlabelled or singly labelled compound.

t.l.c. (silica gel, CH_2Cl_2) R_f = 0.5

^1H NMR (400 MHz, $CDCl_3$) δ 1.28 (quintet 1 : 2 : 3 : 2 : 1, J = 1.0 Hz CH_3-CD_2-O), 2.46 (3H, s, $ArCH_3$) 7.35, 7.80 (2 x 2H, 2 x d, J = 8.0 Hz, Ar-H)

MS m/z 202 (M^+), 172 (M^+-CH_2CD_2), 155 ($CH_3PhSO_2^+$) 108, 91 (CH_2Ph^+)

Measured mass M^+ = 202.0626, calculated for $C_9H_{10}D_2O_3S$ = 202.0633

The intensities of the peaks at m/z 201 and 200 were < 2 % of that of the peak at 202.

CHAPTER 3

SYNTHESIS OF ALKYLCOBALAMINS

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3.1.1 Introduction

A large number of alkyl-cobalt complexes have been synthesised over the past 20 years[145,146]. The cobalt-carbon bond can be formed in one of three ways, summarised in equations 3.1a-c.

Equation 3.1

a) $Co^{I}(chelate)L + R^{+}$
b) $Co^{II}(chelate)L + R\cdot$ $\rightarrow RCo^{III}(chelate)L$
c) $Co^{III}(chelate)L + R^{-}$

R = alkyl group L = α-ligand

The first method, reductive alkylation, is the most widely used, as cobalt(I) species react rapidly with a wide range of alkylating agents[146], including alkyl halides and tosylates, but also with alkenyl halides, activated double bonds, acetylenes, epoxides, and, when in acidic solution, simple olefins[34]. Cobalt(I) nucleophiles are more nucleophilic (at least towards iodomethane) by several orders of magnitude than most conventional nucleophiles (I^{-}, R_3P etc.) and are therefore known as 'supernucleophiles'[147]. The equatorial ligand does not have a large effect on the nucleophilicity towards iodomethane, so, for instance, cobaloximes (A) and Co(SALEN) complexes (D) (Fig.1.4) can be prepared by the same general methods as cobalamins.

There are important differences, however, between the stabilities of alkylcobalamins and those of organo-cobalt complexes with simpler equatorial ligands, probably arising from the greater steric bulk of the corrin ligand. There are, of course, other differences between cobalamins and their models, which are summarised in the Introduction, (Chapter 1).

3.1.2 Steric distortion affects the stability of the Co-C bond

While primary alkylcobalamins can usually be prepared by reductive alkylation in neutral or basic solution, and can be isolated as reasonably stable compounds, secondary alkylcobalamins seemed very unstable and for a long time it was thought that they could not be isolated. This was in contrast to the experience with cobaloximes, as cobaloxime(I) reacts readily with secondary alkyl halides and the secondary (and in some cases, strained tertiary[148]) alkylcobaloximes can be isolated. In 1968 isopropylcobinamide[149] was isolated as a solid which was found to decompose slowly in solution. The authors attempted to make isopropylcobalamin, but were not successful. Cobalamin(I) reacts with secondary alkyl halides under these conditions to produce olefins and desalkylcobalamin. In the presence of excess alkylating agent, a cobalt-catalysed generation of olefins takes place. It seems, therefore, that a transiently formed secondary alkylcobalamin rapidly undergoes β-elimination to give hydridocobalamin and an olefin. The same effect is seen in the reaction of cobaloxime(I) with t-butyl chloride, where isobutene is produced.[34]

An attempt was made to make cyclohexylcobalamin[150], but this was successful only when the 5,6-dimethylbenzimidazole group was quarternised to prevent it from coordinating to the cobalt atom. Cyclohexylcobinamide is isolable, so it seems that the 5,6-dimethylbenzimidazole base is a labilising influence. This was supported by the fact that if cobalamin(I) is produced in acid solution (e.g. by the action of zinc in acetic acid), secondary alkylcobalamins can be produced and can be isolated by precipitation with ether[118]. The UV spectra of these cobalamins is similar to those of the corresponding alkylcobinamides, and so it is apparent that they exist in the protonated 'base-off' form. They also have a characteristic

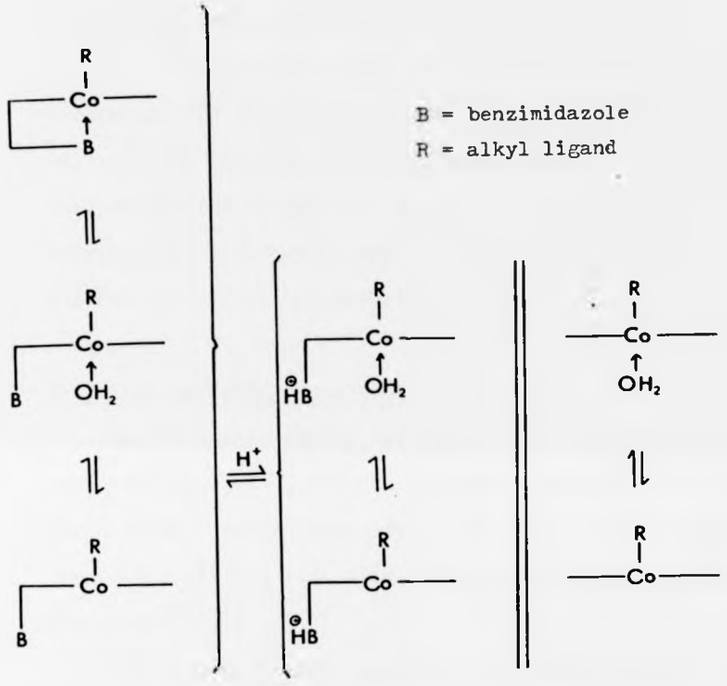
yellow-orange colour, rather than the red of the base-on-forms.

The labilising of the Co-C bond by the axial base could be due to one or both of two effects. Firstly, the coordination of the base may pull the cobalt atom down towards the plane of the equatorial ligand, which in turn will increase the steric compression between the alkyl ligand and the groups on the corrin. Secondly, coordination of the base may cause a conformational change in the equatorial ligand, pushing the group around the periphery of the corrin up towards the alkyl ligand, again labilising the Co-C bond. Detailed information about the conformation of cobalamins in solution is hard to obtain, but it is probable that a combination of these effects is operating. The crystal structure of cobyric acid shows that the equatorial ligand is less puckered than that of adenosylcobalamin[33], which is 'base-on'.

The equilibrium between 'base-on' and 'base-off' forms of alkylcobalamins has been studied by Pratt, Hill *et al.*[149], (Scheme 3.1), who showed that while unhindered primary alkylcobalamins exist predominantly in the 'base-on' form in neutral solution, secondary alkylcobalamins are mostly in the 'base-off' form. Neopentylcobalamin[151,152](54), and some alicyclic alkylcobalamins have contributions from both forms in neutral solution.

The pKa of the axial base is therefore dependent on the nature of the alkyl ligand. That of ethylcobalamin[153] is 3.86, cyclopropylcobalamin is 3.71, cyclobutylcobalamin is 3.83, adenosylcobalamin is 3.35, but cyclohexyl-, cycloheptyl- and cyclooctylcobalamins all have a pKa[118] of about 4.7, the same as that measured for free 1-β-D-ribosyl-5,6-dimethylbenzimidazole[154]. So, the greater the affinity of the axial base for the cobalt atom, the further the pKa is depressed below that of the free base.[154]

Scheme 3.1 Equilibria between base-on and base-off forms of cobalamins and cobinamides (ref. 149)



Thus, in general, it has been found that while simple primary alkylcobalamins can be synthesised and isolated under a wide range of conditions, more hindered cobalamins are much less stable, and special precautions must be taken in order to isolate them successfully. In particular, the pH has to be controlled carefully, as neopentyl-type and secondary alkylcobalamins are only stable at low pH, when the 5,6-dimethylbenzimidazole group is protonated, and so not coordinated to cobalt. Removal of the axial base allows the corrin to bend downwards and so relieves the steric interactions between the corrin and the alkyl β-ligand.

3.1.3 Reduction of hydroxocobalamin to cobalamin(I)

Many reducing agents have been used to reduce cobalt(III) to cobalt(I) species[145], but the one most commonly used is sodium borohydride. Other successful methods used in the reduction of cobalamins include zinc dust, certain dithiols, or controlled potential reduction[145,156].

The reduction takes place in two stages. First, the red cobalamin(III) solution becomes brown cobalamin(II), followed by a further colour change to blackish-green (appearing maroon in fluorescent light) indicating the formation of cobalamin(I). Using borohydride alone, the reduction may stop at the cobalt(II) stage, and so a catalytic amount of cobalt(II) nitrate is added. The mechanism of the cobalt-catalysed borohydride reduction is not clear, although it has been studied by Heinsman and Ganem[157], who propose that in the reduction of nitriles, at least, the active species is the solid cobalt boride (Co_2B) which is formed by the reaction of cobalt(II) ions and borohydride. The hydrogen donor remains unclear, but it is not hydrogen, although this is produced steadily during

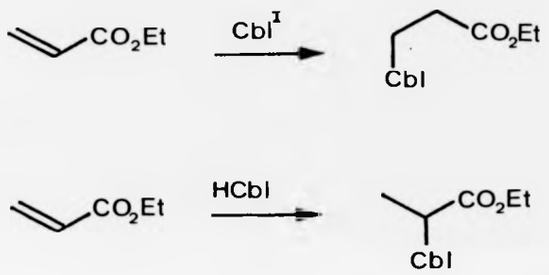
the reaction. It may be free BH_4^-, or it may be some complex formed on the surface of the boride, such as the highly reactive BH_3, bridged borohydrides, or even Co-H species.

In the present work, Co^{2+}-catalysed borohydride reduction was used in the synthesis of primary, unhindered, alkylcobalamins. Neopentyl-type and secondary alkylcobalamins, which are less stable, as explained in the previous section, were prepared by the use of zinc dust in acetic acid or in a methanolic solution of ammonium bromide. These ensure that the axial base is protonated, so the cobalamin is isolated in the more stable base-off form.

Reduction of hydroxocobalamin by zinc in anhydrous acetic acid under anaerobic conditions produces hydridocobalamin[34] which is the protonated form of cobalamin(I). It reacts with alkyl halides to give alkylcobalamins, but it is also apparently more reactive than the unprotonated form, reacting with unactivated double bonds to give primary and secondary alkylcobalamins. For instance Schrauzer *et al.*[34] reported the synthesis of cyclohexylcobalamin from hydridocobalamin and cyclohexene. Hydridocobalamin has not been well characterised, although the UV spectrum has been reported[34], but its pKa has been estimated[158] to be 1.0, in contrast to the value of 10.5 for hydrido(tri-n-butylphosphine)cobaloxime[159]. Its existence has been questioned[35], as the UV spectrum shows only small differences from that of the cobalamin(I) which may be attributed to solvent shifts instead of the large differences expected on protonation of the cobalt, but its reactivity towards activated olefins[34] shows differences from that of free $Co^{(I)}$ nucleophile. For instance, cobalamin(I) reacts with ethyl acrylate to give β-carbethoxyethyl-cobalamin, while hydridocobalamin gives a species which is probably the secondary α-isomer (Scheme 3.2). This parallels analogous

reactions with cobaloximes[159]. Therefore, even if the spectrum reported[34] is not that of hydridocobalamin alone, but of a large proportion of cobalamin(I), it is possible that hydridocobalamin is present as a transient intermediate, and is the reactive species.

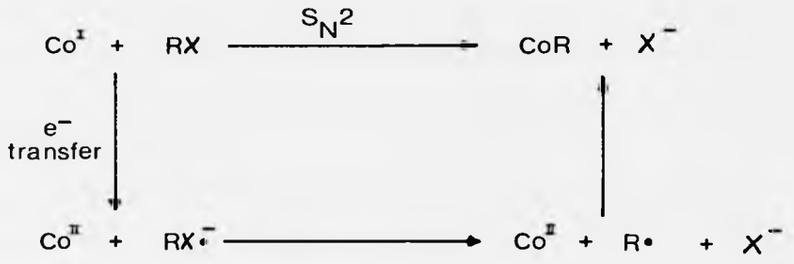
Scheme 3.2 Reaction of ethyl acrylate with cobalamin(I) and hydridocobalamin (ref. 34).



3.2 Mechanism of alkylation

The cobalamin(I) nucleophile can be alkylated in one of two ways (Scheme 3.3). It can either react by S_N2, with direct displacement of the leaving group from the alkylating agent, and inversion of the carbon centre, or it can undergo electron transfer, giving rise to an intermediate alkyl radical and cobalamin(II), which combine to give the alkylcobalamin.

Scheme 3.3



Halpern[160] showed that certain bis(dioximato)Co^{II} complexes react with alkyl halides according to equations 3.2 (i)-(iii).

Equation 3.2

(i) $Co^{II}(chelate)_2B + RX \rightarrow XCo^{III}(chelate)_2B + R$

(ii) $Co^{II}(chelate)_2B + R \rightarrow RCo(chelate)_2B$

overall

(iii) $2Co^{II}(chelate)_2B + RX \rightarrow XCo(chelate)_2B + RCo(chelate)_2B$

Equation 3.2(i) is rate determining, and the reaction rate is second order (rate α $[Co^{II}(chelate)_2B][RX]$).

The rate also depends on the nature of the alkyl halide RX, with $k_{RI} > k_{RBr} > k_{RCl}$, and the rate of reaction of secondary alkyl halides is faster than that of primary ones. Also, electron-withdrawing substituents increase the rate, while there is only a small dependence on solvent polarity. All these observations and the overall observed stoichiometry led the authors[160] to postulate

the reactions in Equations 3.2, rather than any other mechanisms, such as those involving disproportionation of cobalt(II) species to cobalt(I) and cobalt(III), or electron transfer from a 6-coordinate cobalt complex ($Co(chelate)_2B_2$) to the alkyl halide in the rate determining step.

A similar reaction has been observed[161] with cobalamin(II) (Equation 3.3).

$$2Cbl(II) + RX \longrightarrow R\text{-}Cbl + XCbl[\overset{H_2O}{\rightleftharpoons} OHCbl] \qquad (3.3)$$

and for other cobalt complexes.

These results show that cobalt(II) complexes react with certain alkyl halides by transfer of an electron to give an alkyl radical and a halide ion, as well as the resulting cobalt(III) species. The alkyl radical can then combine with a further molecule of the cobalt(II) complex, to give an organo-cobalt(III) complex, and the halide ion coordinates to the cobalt(III) species.

In the case of cobalamin(I), a similar reaction could in theory occur, with single electron transfer from cobalt to give an alkyl radical, a halide ion and cobalamin(II). The alkyl radical could then combine with cobalamin(II) to give an alkylcobalamin. Studies by Schrauzer and Deutsch[162], however, showed that the dependence of the relative rates of reaction of Co^I-nucleophiles on the structure of the alkyl group, is similar to that of S_N2 reactions, and quite distinct from that for free radical reactions. Also, the rate is increased by substituent groups which can stabilise charge in the transition state (-CN, CH_3O-, Ph-, etc.). Jensen *et al.*[163] showed that substituted cyclohexyl bromides and tosylates react with cobaloxime(I) with inversion of configuration at the displacement centre. These studies were thought to show that cobalamin(I) reacts

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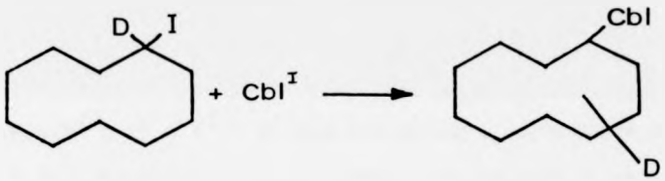
by S_N2 mechanism.

Nevertheless, there are exceptions to this. The formation of 1-adamantyl- and 1-norbornyl(pyridine)cobaloximes[148] must proceed with retention, so the electron-transfer mechanism seems likely for these cases. It has also been found[164] that cyclodecyl-1-^{2}H-tosylate does not react with cobalamin(I), but cyclodecyl-1-^{2}H-iodide does react to produce cyclodecylcobalamin with the deuterium distributed over various positions in the ring. (Equation 3.4).

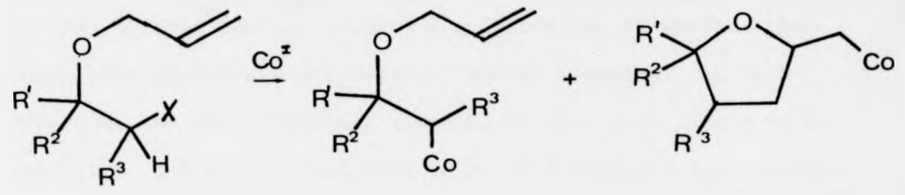
Equation 3.4



This is taken to be evidence that the iodide initially undergoes electron transfer and the resulting radical can transfer hydrogen across the ring before combination with cobalamin(II). The failure of the tosylate to react is consistent with this as tosylates do not take part in electron transfer reactions.

The work of Tada and Okabe[165] has supported these findings. In the reaction of cobaloxime(I) with substituted 2-(allyloxy)ethyl halides and tosylates they showed that radical intermediates were likely in the case of bromides and iodides. Equation 3.5 shows the general reaction.

Equation 3.5



A B

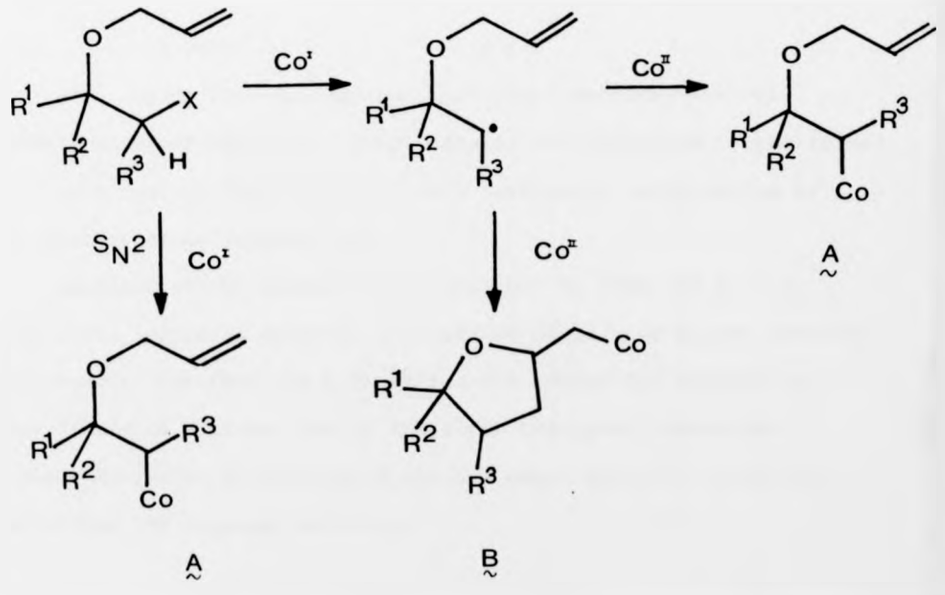
When $R^1 = R^2 = R^3 = H$, and X = Br or I, a mixture of products A and B was obtained. With X = OTs, only product A was found. If one or more of R^1, R^2 and R^3 was substituted as phenyl or alkyl, when X = Br or I, only products corresponding to B were obtained, and no reaction occurred when X = OTs.

The cyclised products B were evidence for the intermediacy of a radical species, while the non-cyclised products could arise by S_N2 or direct coupling of the radical with cobaloxime(II) before it rearranges (Scheme 3.4). The fact that 2-(allyloxy)ethyl tosylate gave only the direct substitution product A suggests that S_N2 is the mechanism here. The tosylates that are substituted at the α or β positions did not react with cobaloxime(I), probably because of steric inhibition. Corresponding substituted bromides and iodides can undergo electron transfer, and are found to give exclusively the cyclised products B. The steric interactions that slow down S_N2, are also likely to retard the direct coupling of radicals to

give the uncyclised product, and so the less crowded, cyclised product is formed.

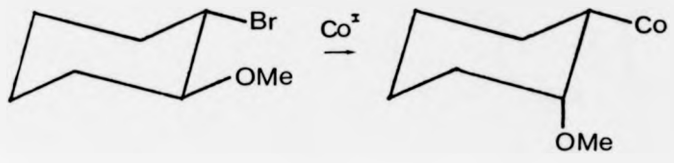
In the case of unsubstituted 2-(allyloxy)ethyl bromide and iodide, where a mixture of products was obtained, studies were done to show whether the S_N2 process was operating, or whether the uncyclised product arose by direct radical coupling. At high dilutions, the direct radical coupling to give A is likely to be negligible and so the formation of A (40 % from 2-(allyloxy)ethyl bromide) must be by S_N2. In all cases, the iodide gives more cyclised product than the bromide, indicating that the electron transfer step is rate determining, and that there is competition between S_N2 and electron transfer, at least in the case of the unsubstituted bromide.

Scheme 3.4



Tada and Okabe[166] also show that some of the results of Jensen *et al.*[163] with cyclohexyl bromides can be explained by the electron transfer mechanism rather than S_N2. They point out that while the cobaloxime which is obtained appears to be the product of inversion (Equation 3.6), it is only obtained in low yield (< 30 %), and other products were also formed.

Equation 3.6

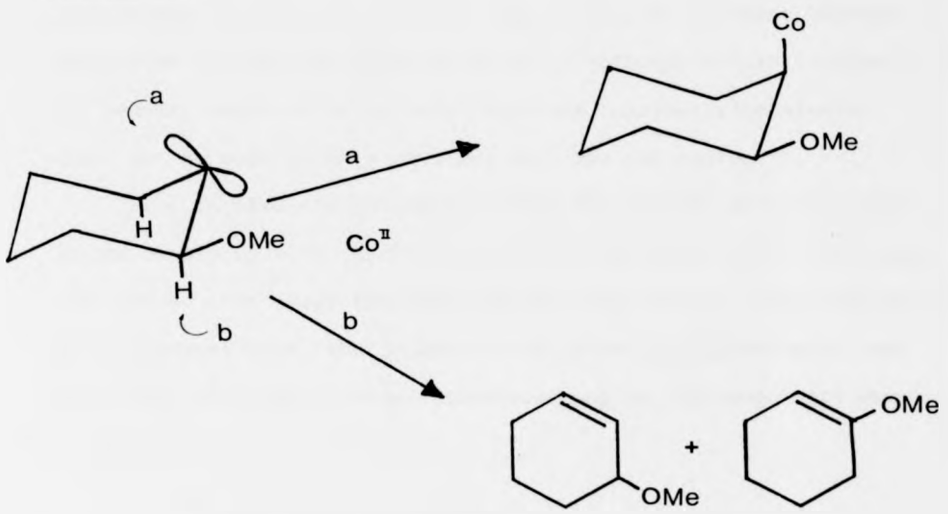


(trans) *(cis)*

Also, using the *cis*-2-methoxycyclohexyl bromide, the *cis*-product is again obtained. These results are explained by the formation of a radical that is locked in a particular conformation by the methoxy group (Scheme 3.5)

Approach of the cobalt(II) species may be from the a or b direction. Approach from the a direction gives rise to the observed *cis*-product, but from the b direction the cobalt(II) species is more likely to abstract one of the axial hydrogens, which are already activated by overlap of the C-H bonds with the p-orbital containing the unpaired electron.

Scheme 3.5



In summary, it seems that although the cobalt(I) nucleophile can be considered as a 'supernucleophile' in its reactions towards unhindered species such as iodomethane, its 'soft' character, and the steric interactions caused by the large equatorial ligand systems, mean that this nucleophilicity is not shown towards hindered alkylating agents. Instead, electron transfer becomes more important.

Monosubstituted epoxides react with cobalamin(I) to give β-hydroxyalkylcobalamins, presumably by an S_N2 mechanism.

In practical terms, this means that while tosylates and other sulphonate leaving groups can be used in the synthesis of primary, unhindered alkyl cobalt species, in order to make secondary or β-substituted alkyl cobalt compounds, bromides or, preferably, iodides must be used. This was demonstrated in the present work where attempts to make cycloalkylcobalamins from the corresponding tosylates were

not successful. It was hoped that trifluoromethanesulphonate derivatives (triflates) could be used as this is a better leaving group than tosylate by a factor of 10^5. Although n-hexylcobalamin was readily obtained using hexyl triflate, cyclooctylcobalamin could not be made in this way, but only *via* the iodide.

There is also the possibility that the radical will rearrange before combining with cobalt(II), so the structure of the cobalamin will not be that predicted from the structure of the alkylating agent. In the present work, the ^{1}H NMR spectra of the alkylcobalamins were consistent with the expected structures and no rearrangements were detected.

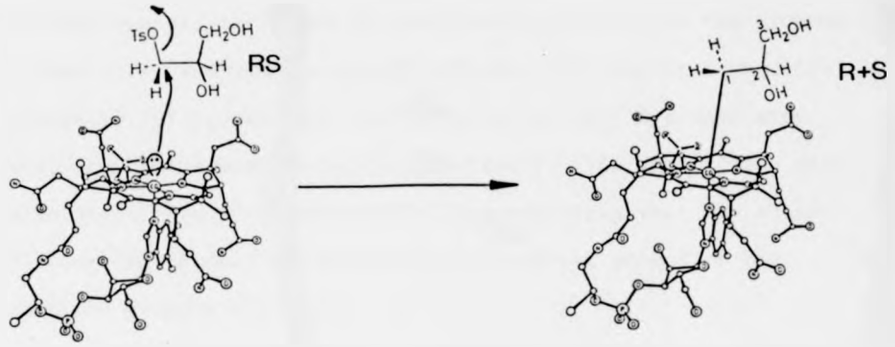
3.3 Enantioselectivity of cobalamin(I) towards racemic alkylating agents

3.3.1 General considerations

When cobalamin(I) reacts with a racemic alkylating agent, a pair of diastereoisomers may be formed (Scheme 3.6). Since the rate of reaction with each enantiomer can be different, the diastereoisomers may be formed in unequal amounts. Part of the present work was to look at the enantioselectivity of cobalamin(I). The reasons for this were twofold.

First, enzymic reactions are well-known to be highly enantioselective[167]. Vitamin B_{12}-dependent enzymes are, in general, no exception. For instance glutamate mutase[168] reacts with L-glutamate but not D-glutamate. Propanediol dehydratase is unusal in this respect, in that it accepts both (*R*)-and (*S*)-propane-1,2-diol as substrates. This is not to say that it does not distinguish between them. In fact, (*R*)-propane-1,2-diol reacts 1.5 to 2 times as fast

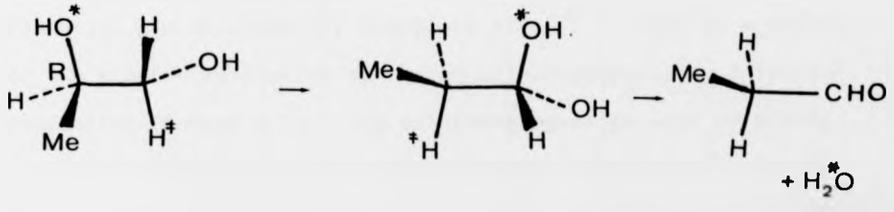
Scheme 3.6



as the (*S*)-isomer[169], when the two substrates are presented separately, but when racemic propanediol is used, the (*S*)-isomer reacts preferentially. These results have been interpreted[169] to show that (*S*)-propanediol binds more readily to the enzyme, but, once bound, (*R*)-propanediol reacts faster.

Although the enzyme lacks a rigorous enantioselectivity in accepting substrate molecules, the rest of the rearrangement reaction (e.g. Equation 3.7) proceeds with a high degree of stereospecificity[66,70].

Equation 3.7



These studies are described in more detail in the Introduction (Section 1.7.3.) but, in summary they show that the rearrangement occurs with overall inversion of configuration at C-2 of the propane-1,2-diol molecule, and that the pro-(*R*) hydrogen atom migrates when the substrate is (*R*)-propane-1,2-diol, and the pro-(*S*) hydrogen atom migrates when the substrate is the (*S*)-isomer. The dehydration step was also shown[72] to be stereospecific, demonstrating that the enzyme can distinguish between the enantiotopic hydroxyl groups of the intermediate propane-1,1-diol.

Depending on the structure of the active site of the enzyme, this enantioselectivity may be largely due to the enzyme itself, or it may be contributed to by the cobalamin, especially if the substrate comes into close contact with the cobalamin as suggested in certain mechanistic schemes[101]. Therefore it was of interest to see what degree of enantioselectivity is exhibited by cobalamin(I).

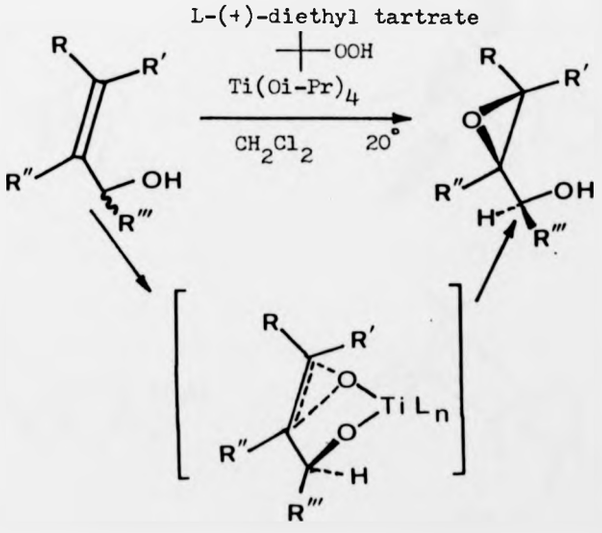
The second reason was the interest in searching for enantioselective reagents for use in organic synthesis. Until recently the only such reagents with high enough enantioselectivities to be of practical use were enzymes. These could be used for the kinetic resolution of a limited number of substrates. For instance, pig kidney deacylase reacts only with L-*N*-acylvaline, and so racemic DL-*N*-acylvaline can be separated into D-*N*-acylvaline and L-valine by this enzyme[170].

One of the very few efficient non-enzymic methods of kinetic resolution was developed by Sharpless *et al.*[171]. This is a method for the kinetic resolution of chiral allylic alcohols by selective epoxidation (Scheme 3.7). The oxidising agent is made by mixing titanium tetraisopropoxide, t-butylhydroperoxide and (+) or (-)-diisopropyl tartrate, depending on which enantiomer of the allylic

alcohol is desired. The enantioselectivity of this system was enough to give relative rates of epoxidation k_{fast}/k_{slow} > 100 for certain pairs of enantiomers. The configuration of the oxidising agent was chosen to be the one that reacted more slowly with the desired enantiomer. The reaction was allowed to proceed until a calculated proportion (usually 55-60 %) of the alcohol had been consumed, and the recovered unreacted alcohol would be greatly enriched in the desired enantiomer. Enantiomeric excesses of 90 to > 95 % were achieved by this method. By allowing the reaction to proceed further, any desired enantiomeric excess could theoretically be achieved, although at the expense of the absolute yield, so long as k_{fast}/k_{slow} was known.

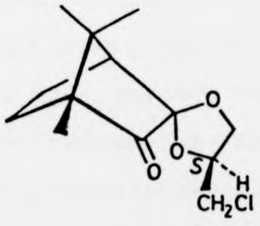
Kinetic resolution depends on the difference in energy between the diastereomeric transition states, which is seen as a difference in the rate of reaction of the two enantiomers.

Scheme 3.7 Kinetic resolution of allylic alcohols by Sharpless epoxidation (ref. 171).

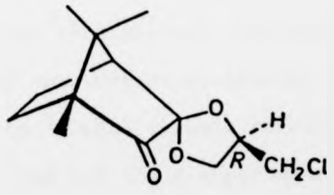


An interesting feature of this reaction is that the final enantiomeric purity does not depend on the enantiomeric purity of the diisopropyl tartrate used to make the titanium complex, although this sets a limit on the k_{fast}/k_{slow}. Therefore the reaction has to be allowed to proceed further if the tartrate is not 100 % optically pure.

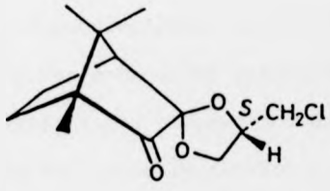
A kinetic resolution of 1,2-diols using D-camphorquinone was recently developed by Ellis *et al.*[141]. D-Camphorquinone reacted with 1,2-diols to give a mixture of four diastereoisomeric ketals (55 a to d). The proportion of these isomers was under kinetic control. In the case of (*rac*)-3-chloropropane-1,2-diol, a slight selectivity was shown towards the (*R*)-isomer (55 b + 55 d):(55 a + 55 c) = 57 : 43). The major isomer (55 b) (48 %) could be separated by fractional crystallisation, and the other isomers could be separated by preparative HPLC. The pure enantiomers of the 1,2-diol could be regenerated by hydrolysis.



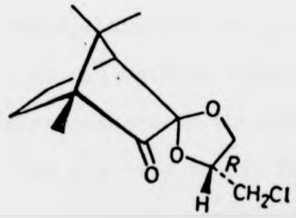
(55 a)



(55 b)



(55 c)



(55 d)

Reactions of the epoxides with chiral reagents have also been studied[172]. Protected amino acids showed no enantioselectivity towards methyloxirane, and of all those tested, only valine methyl ester showed a significant enantioselectivity towards t-butyloxirane.

It was therefore of interest to investigate the reaction of cobalamin(I) with various epoxides and other alkylating agents.

3.3.2 Reaction of cobalamin(I) with racemic alkylating agents

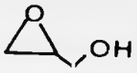
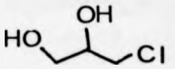
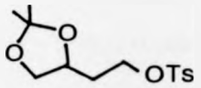
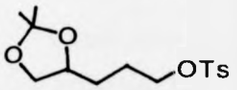
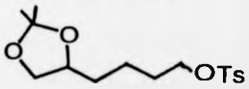
To this end, a series of monosubstituted epoxides were prepared (I) to (IV) (Table 3.1). Monosubstituted epoxides are known to react with cobalamin(I) at the less hindered end to give β-hydroxy, primary alkylcobalamins.

The epoxides were reacted with cobalamin(I) in 50 % aqueous methanol, which ensures a homogenous reaction mixture. A > 10-fold excess of the epoxide was used, so that the diastereomeric ratio reflected the true difference in reaction rates, and both enantioners remained in excess. The resulting alkylcobalamins were isolated by the usual method of thorough extraction into phenol, then back into water, and were freeze-dried. They were not crystallised, so as not to change the diastereomeric ratio. Other racemic alkylating agents were also used, and the results from all these experiments are summarised in Table 3.1.

The ratio of diastereoisomers was estimated by integration of corresponding peaks in the high-field NMR spectrum of the mixture. No other methods of analysis (IR, UV, t.l.c. etc) could be found that distinguished between the diastereoisomers, except in the special case described in section 3.3.2, where FAB m.s. was used. The differences in the 400 MHz ^{1}H NMR spectra of diastereomeric cobalamins

Table 3.1

Enantioselectivity of cobalamin(I) towards racemic alkylating agents

Alkylating agent	no.	yield	Diastereomeric ratio *R* : *S*	R_{CN}*
	I	52 %	3 : 1	1.2
	II	-	1.8 : 1	0.8
	III	-	1.3 : 1 (a)	1.4
	IV	88 %	4.3 : 1 (a)	1.5
	V	58 %	1.1 : 1	0.8
	VI	75 %	1.0 : 1	0.9
	VII	-	1.0 : 1	1.6
	VIII	-	1.0 : 1	1.6

(a) ratio may be (*S*:*R*) rather than (*R*:*S*)

* $R_{CN} = \frac{R_f(RCbl)}{R_f(CNCbl)}$ (t.l.c.; cellulose, solvent A)

are discussed in Chapter 4. In general, cobalamins with the chiral centre in the β-position to cobalt, showed the largest differences, with almost all the resonances showing at least slight differences between the (*R*)- and (*S*)-forms. Some resonances showed differences of up to 0.4 ppm. It was usually quite straightforward to find suitable resonances to integrate to obtain the diastereomeric ratio.

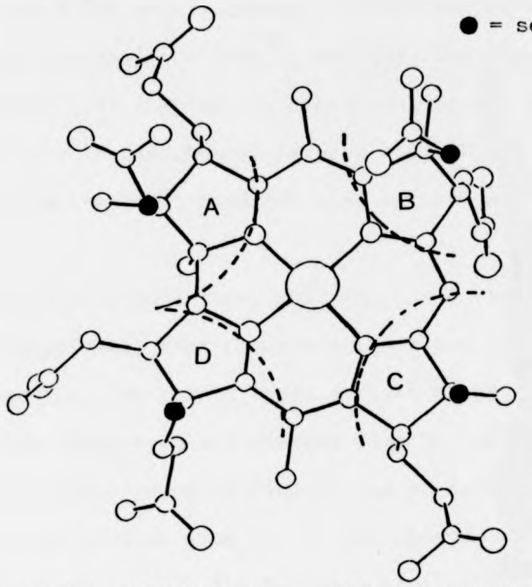
Cobalamins such as 3,4-dihydroxybutylcobalamin and 4,5-dihydroxypentylcobalamin, with the chiral centre at the γ- and δ-position respectively, showed less marked differences between the diastereoisomers, but the resonances of the protons on the alkyl ligand could still be distinguished and integrated to give the required ratios.

3.3.3 Discussion

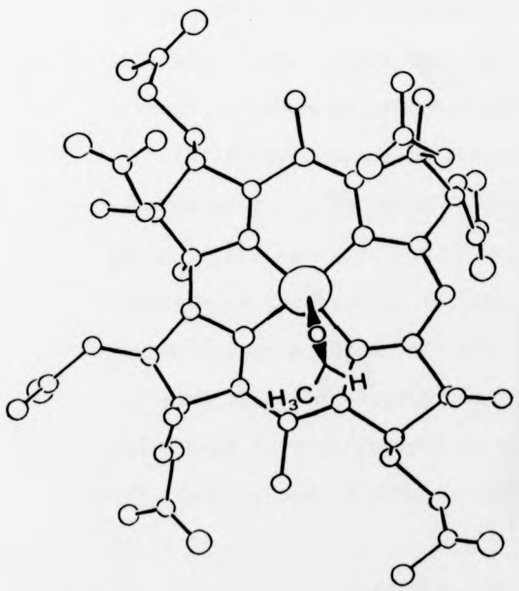
Table 3.1 shows that cobalamin(I) is moderately enantioselective, with, in general, a slight increase in selectivity with more bulky alkylating agents with the chiral centre near to the site of attack. Nevertheless, the greatest selectivity shown was only 4.3 : 1 with (*rac*)-t-butyl oxirane. In this case, crystallisation from aqueous acetone increased the ratio to 5.4 : 1. No selection at all was shown when the chiral centre was further than two carbon atoms away from the leaving group.

In the cases where enantioselectivity was shown, and the pure diastereoisomers were available in order to determine which isomer was (*S*) and which was (*R*), the major isomer was always (*R*). This can be rationalised as follows. Scheme 3.8a shows a view of cobalamin(I) from the β-side, that is, from the direction from which the alkylating agent approaches. X-ray crystal studies have shown that the cobalt atom is protected on this face by four 'sentinel groups', one on each of rings A to D. Those on rings A and B are acetimido- and

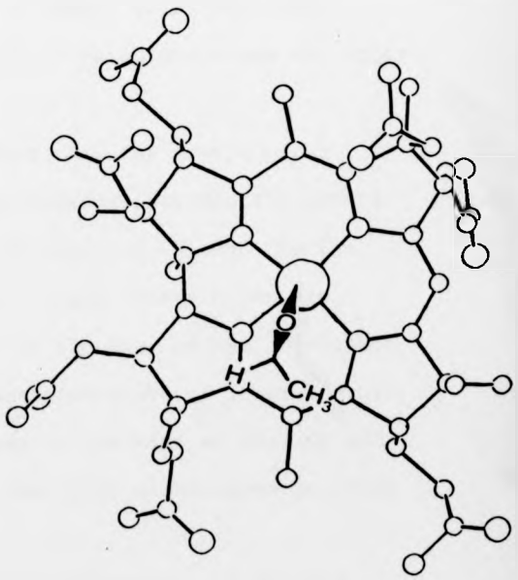
Scheme 3.8 a β-side of cobalamin



3.8 b Approach of S-methyloxirane



3.8 c Approach of R-methyloxirane



those on C and D are methyl groups. Inspection of the crystal structures of adenosylcobalamin[33], and (*R*)- and (*S*)-2,3-dihydroxypropylcobalamin (see chapter 5), shows that in all these cases the alkyl group lies in the channel between rings C and D. This then, as expected, is the least hindered line of approach to the cobalt atom.

Inspection of models shows that the A - D ring junction is much more crowded than that between rings B and C. When (*S*)-methyloxirane approaches the cobalt atom, it lies along the C - D channel and the methyl group projects towards ring D. It therefore interacts with the methyl group on ring D, and probably also with the hydrogen atom on C-19 of ring D. If the epoxide rotates to avoid these steric interactions, the hydrogen atom on C-1 of the epoxide is brought close to the methyl group on ring C (C12β-CH_3) and probably C13-H. A possible line of attack which minimises the interactions is shown in Scheme 3.8b.

(*R*)-Methyloxirane, on the other hand, has the methyl group towards ring C. The main interactions here are between the methyl group of the epoxide, and C13-H, and between C17-CH_3 and the C-1 hydrogen of the epoxide (Scheme 3.8c). These interactions are presumably more favourable than those in the case of the (*S*)-isomer, and give rise to a diastereomeric transition state of lower energy. This leads to a difference in the rates of reaction of the (*R*) and (*S*) isomers, and in turn is shown in the (*R*:*S*) diastereomeric ratio of (3:1).

The alkylating agents such as t-butyloxirane (IV) and cyclohexyloxirane (III) might be expected to give much larger diastereomeric ratios than methyloxirane, since t-butyl or cyclohexyl groups might be expected to interact much more strongly with the

cobalamin than the methyl does. In fact, t-butyloxirane shows only a slightly larger ratio than methyloxirane (4.3 : 1 instead of 3 : 1) and cyclohexyloxirane shows very little difference in the amounts of diastereoisomers (9:7). These two epoxides (III) and (IV) were not prepared in optically pure form, so it is not known whether the ratios are (*R*:*S*) or (*S*:*R*), (but see section 4.3.3.c).

There are two possible explanations for this low enantioselectivity. It is possible that the ratios are not a true reflection of the enantioselectivity of cobalamin(I). This could occur if the epoxides were only slightly soluble in the reaction mixture. Although the alkylating agent was added in excess, the cobalamin(I) would be exposed to only a very low concentration, the rest remaining in a separate phase. Then, reaction with one enantiomer preferentially would deplete the reaction mixture of this enantiomer, and if replenishment from the other phase was too slow, the cobalamin(I) would have to react with the slower reacting enantiomer. This would distort the diastereomeric ratio, giving a lower difference than the true one. It was hoped that this problem had been overcome by using 50 % aqueous methanol as the solvent, in which all the alkylating agents seemed to be soluble and which gave homogenous reaction mixtures.

The other possibility is that cobalamin(I) does in fact show a lower enantioselectivity towards the epoxides with large alkyl groups, than might be expected from the results with epoxypropane. This may be because when either enantiomer of cyclohexyloxirane, for instance, comes into the cavity of cobalamin(I) in order to react, the cyclohexyl group, being so large, interacts with the methyl groups both on ring C and on ring D. These interactions, being spread over both rings for each enantiomer, give a low enantio-

selectivity. Methyloxirane, in contrast, experiences very different interactions for each enantiomer, since the methyl group is small. t-Butyloxirane gives a larger enantioselectivity than methyloxirane, but not such a difference as might be predicted from the considerable difference in size between a methyl and a t-butyl group. Again, the same effect may be operating, with the large alkyl group experiencing steric interactions with both the C and D rings of the cobalamin(I), which will tend to lessen the differences in energy between the diastereomeric transition states.

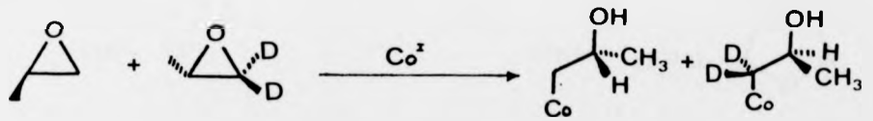
3.3.4 Investigation of diastereomeric ratio by FAB m.s.

In conjunction with M.K. Ellis, who prepared the deuterium-labelled, optically active alkylating agents[172], a study was undertaken to distinguish the diastereoisomers of 2 -hydroxypropylcobalamin by fast atom bombardment mass spectrometry (FAB m.s.).

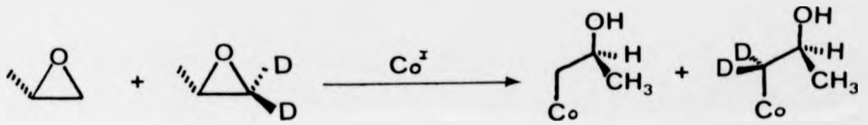
An equimolar mixture of (*S*)-2,2-[$^2H_2$]-methyloxirane and (*R*)-methyloxirane was used to alkylate cobalamin(I) (Equation 3.8a). Method A(i) (see Experimental section) was used, but the cobalamin was not chromatographed or crystallised.

In order to estimate the magnitude of the secondary isotope effect, cobalamin(I) was alkylated with an equimolar mixture of labelled and unlabelled (*S*)-methyloxirane (Equation 3.8b).

Equation 3.8 a

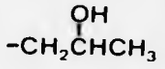
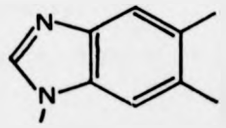
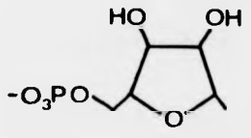


b



The two sample were submitted for FAB m.s. and the results are summarised in Table 3.2. The ratios of diastereoisomers are in qualitative agreement with those found by NMR, but no quantitative calculations could be done because of the large number of overlapping peaks in the mass spectrum.

Table 3.2

	(*R*) + [$^2H_2$]-(*S*)-methyloxirane	&	(*S*) + [$^2H_2$]-(*S*)-methyloxirane
m/z	ion		ion
1390	$^2H_2$-M+		$^2H_2$-M+
1388	M+		M+
1331	M- 59		M- 
1184	1331- 147		1331- 
971	1184- 213		1184- 

3.4 Preparation of alkylcobalamins by reductive alkylation

The general method is summarised in Equation 3.9 but, depending on the stability and properties of the resulting organo-cobalt derivative, the details of solvent, reducing agent, alkylating agent and method of isolation can be varied.

Equation 3.9 $Cbl^{III} \xrightarrow{\text{"2e"}} Cbl^{I} \xrightarrow{RX} RCbl + X$

In the following sections, three general methods are discussed. Detailed methods for the preparation of particular cobalamins are given in the Experimental Section (3.6).

3.4.1 Synthesis of alkylcobalamins at high pH (Method A)

Most primary cobalamins are stable in solution in the dark at the high pH values (*ca* pH 12) which are found in reaction mixtures which use sodium borohydride as the reducing agent. This method was found to be easy and convenient, and was used in the preparation of most of the primary alkylcobalamins, except for neopentyl-type cobalamins. These are not stable in alkaline solution, because of their steric bulk, as outlined in Section 3.1.2.

The method described follows closely that of Dolphin[156], although modifications have been made to suit the particular cobalamins being prepared.

Hydroxo- or cyanocobalamin was dissolved in water, or a mixture of water and methanol or ethanol. The choice of solvent was determined by the solubility of the alkylating agent. A minimum volume of methanol was used, however, to make the work-up of the preparation

easier and to avoid the formation of emulsions during extractions. A catalytic amount of a cobalt(II) salt was added to catalyse the reduction (Section 3.1.3), and the solution, in a serum capped conical flask, was deoxygenated by the passage of a stream of argon through the solution.

Reduction of cobalt(III) to cobalt(I) was achieved by the injection of a solution of sodium borohydride (> 5-fold excess). The reaction mixture was seen to change colour rapidly from red to brown, then more slowly to blackish-green, with the evolution of hydrogen. The excess of sodium borohydride seemed necessary for rapid reduction, and to compensate for that which may decompose by reaction with the solvent.

The blackish-green colour indicated that cobalamin(I) was formed, and the reaction vessel was protected from light. Argon flushing was continued and the alkylating agent (5 to 10-fold excess, if available) was added *via* syringe, neat, or in deaerated solution in methanol. The reaction mixture rapidly turned colour from black to red, showing that the alkylcobalamin had formed. Since the cobalt-carbon bond is unstable to light in solution, all further manipulations were carried out in the dark, or under a red photographic safe-light (*e.g.* Wrattan filter 1A), and reaction vessels were shielded with aluminium foil. The reaction was usually complete within a few minutes, and the completeness could be assessed by t.l.c. of a portion of the reaction mixture. In one experiment, samples were taken at 5, 10, 15, 20 and 30 min after addition of the alkylating agent, and tested by t.l.c. Conversion into the alkylcobalamin was complete within the first five minutes, and no increase or decrease in yield was detected. If the organo-cobalt derivative was left in contact with borohydride, it might be expected to decompose by reductive

cleavage of the Co-C bond. Therefore the remaining borohydride was destroyed by the addition of a few drops of acetone after not more than 30 min.

Hydrolysis of acetals (Method Aii)

Dihydroxy-n-alkylcobalamins (except for dihydroxypropylcobalamin) were prepared from the corresponding *O*-isopropylidene derivatives, to avoid the danger of intramolecular displacement of the leaving group by a free hydroxyl group. The acetals were hydrolysed by stirring the cobalamins in dilute hydrochloric acid and monitoring the reaction by t.l.c. (cellulose, solvent A, section 3.7.1). The acetals had $R_{CN} > 1$, whereas the dihydroxyalkylcobalamins had $R_{CN} < 1$.

3.4.2 Isolation of stable cobalamins

Stable, primary alkylcobalamins were isolated by Dolphin's[156] methods, in which they were extracted out of the aqueous reaction mixture into a phenol : dichloromethane mixture (100 g in 100 ml). After dilution of the organic phase with dichloromethane, the cobalamin was extracted back into water, washed with dichloromethane, and freeze-dried, to give the alkylcobalamin as a feathery, amorphous, red solid. At this stage, hydroxocobalamin could sometimes be detected as a contaminant, and this could be removed by passage down a short column of t.l.c.-grade silica gel, using n-propanol : water : NH_3 (100 : 99 : 1)[220] as an eluent. Hydroxocobalamin remained at the top of the column and the alkylcobalamin was eluted.

Ion-exchange chromatography[173], using a carboxymethylcellulose column (Whatman CM52), eluting with water, was found to require very slow running columns and the cobalamins were recovered in large volumes of solvent (see Section 3.7.3).

Alkylcobalamins were crystallised from aqueous acetone. A

concentrated aqueous solution was prepared (*ca* 25 mg/ml) and AR acetone was added until a faint cloudiness persisted (about 7 volumes were usually required). The solution was left overnight at room temperature, and needle-shaped crystals were deposited. These were collected by filtration, washed with a little 90 % acetone/water, followed by AR acetone, and dried in a stream of nitrogen. Cobalamins were stored at -20 ° in tightly-stoppered, foil-wrapped vials, although storage at room temperature for a few days was not found to lead to decomposition.

3.4.3 Preparation of alkylcobalamins at low pH (Method B)

This method is based on that of Schrauzer *et al.*[152] and was used to prepare neopentylcobalamin and others of a similar type. A 5 % solution of ammonium bromide in methanol was used as the solvent. This was acidic enough to allow protonation of the 5,6-dimethylbenzimidazole group, and so ensure that the cobalamin was largely 'base-off'.

Activated zinc dust was used as the reducing agent, and the reaction was done in a serum-capped centrifuge tube, which was deoxygenated with argon. The colour change from red to brown to blackish-green was similar to that seen with borohydride reductions, then the alkylating agent was injected into the mixture.

After 2-3 min, the tube was centrifuged to remove the zinc powder, which decomposes alkylcobalamins, if they are allowed to stay in contact for too long. The supernatant was poured into 1 M HCl, then the cobalamin was extracted into a minimum volume of phenol : CH_2Cl_2, using small portions until the phases were colourless. The dichloromethane was evaporated and the phenolic cobalamin solution was poured into dry acetone : diethyl ether (1:9). The precipitated

cobalamin was collected by centrifugation, washed several times with dry ether, and dried with a stream of dry nitrogen.

3.4.4 Preparation of secondary alkylcobalamins (Method C)

This method is also based on a method of Schrauzer[118]. Secondary alkylcobalamins are very unstable, so care is taken to work in the lowest possible light intensity, and to leave the cobalamins in solution for the shortest possible time.

The solvent was glacial acetic acid, and the reducing agent was zinc powder, which could be conveniently removed by centrifugation when the reaction was complete. Flushing with argon of the serum-capped centrifuge tube which contained the cobalamin in glacial acetic acid, and zinc powder, quickly produced the required colour change to black, showing that cobalamin(I) was present. The alkylating agent was injected, and after 1 minute, the tube was centrifuged. The supernatant was poured into dry ether, which precipitated the yellow-orange, 'base-off' cobalamin, but left the contaminants, including zinc salts, in solution. The cobalamin was collected as before by centrifugation, washed with dry ether and dried in a stream of dry nitrogen.

The alkylating agent must be an iodide, rather than a tosylate, for the reasons discussed in Secion 3.2. The attempts to use secondary tosylates to alkylate cobalamin(I) did not give alkylcobalamins.

3.5 EXPERIMENTAL SECTION

Typical examples of methods A, B and C for the preparation of alkylcobalamins are given. A complete list of cobalamins and the methods used in their preparation is given in Table 3.3.

3.5.1 Method A: (*R*)-2,3-Dihydroxypropylcobalamin

A solution of hydroxocobalamin (100 mg, 0.07 mmol) and $Co(NO_3)_2$ (1 mg, 0.005 mmol, 8 mol %) in water (10 ml) in a serum-capped, conical flask, was deoxygenated by bubbling a slow stream of argon for 20 min. Sodium borohydride (20 mg, 0.53 mmol) in water (1 ml) was added *via* syringe. The colour of the reaction mixture changed from red to brown to blackish-green, and hydrogen was evolved. After 5 min, (*R*)-glycerol 1-*O*-tosylate (47) (100 mg, 0.4 mmol) was added, in solution in water (1 ml), and the flask was protected from light. The rest of the procedure was done in the dark or under a red safe-light. After a further 30 min, an aliquot of the reaction mixture was tested by t.l.c. (Section 3.7.1) and showed complete conversion into the alkylcobalamin. The reaction was stopped by the addition of acetone (0.5 ml) which reacted with the remaining borohydride.

The cobalamin was isolated by extraction through phenol (section 3.6) and obtained in water (∿ 100 ml). The aqueous solution was freeze-dried, and the crude alkylcobalamin (contaminated with a small amount of hydroxocobalamin) was purified by short column chromatography (section 3.7.2). The pure cobalamin (90 mg, 90 %) was crystallised by dissolving in water (3 ml) and slowly adding AR acetone (∿ 25 ml) until a faint cloudiness persisted). On leaving the mixture overnight, red needle-shaped crystals of (2*R*)-2.3-dihydroxypropylcobalamin (81 mg, 81 %) formed. These were collected by filtration and washed with a small amount of water : acetone (1:9) then with acetone, and were dried in a stream of air or nitrogen. The alkylcobalamin was stored in a tightly-stoppered, foil-wrapped vial at -20 °.

t.l.c. R_{CN} = 0.8 (cellulose, solvent A).

Method A(i) was also used in the experiments to test the enantioselectivity of cobalamin(I) towards racemic alkylating agents. In these cases, the alkylcobalamins were not crystallised, as this could alter the diastereomeric ratio.

3.5.2 Method A(ii). (4*S*)-4,5-Dihydroxypentylcobalamin

The procedure described in method A(i) was followed using cyanocobalamin (250 mg, 0.17 mmol) and cobalt nitrate (5 mg) in ethanol : water (1:1 v:v) (20 ml). Sodium borohydride (50 mg, 13.2 mmol) in water (1 ml) reduced the cobalamin(III) to cobalamin(I), and (*S*)-2.2-dimethyl-4-(3-tosyloxypropyl)-1,3-dioxolan (24) (0.3 g, 0.96 mmol) in ethanol (1 ml) was added. After 30 min acetone (0.1 ml) was added and the solution evaporated to dryness (Rotovap, < 40 °). Hydrochloric acid (0.1 M, 20 ml, prestandardised against *ca.* 0.1 M NaOH) was added, and the solution stirred for 3 h. in the dark. At the end of this time, t.l.c. showed that all the acetal had been hydrolysed (R_{CN}(acetal) = 1.6, R_{CN}(diol) = 0.9), so the mixture was neutralised by the addition of the calculated volume of standard sodium hydroxide solution.

Isolation of the cobalamin was as described in Method A(i). Yield of (4*S*)-4,5-dihydroxypentylcobalamin was 190 mg (76 %). R_{CN} = 0.9 (t.l.c. cellulose, solvent A).

3.5.3 Method B. Neopentylcobalamin[152]

Hydroxocobalamin (125 mg, 0.08 mmol) in a 5 % solution of ammonium bromide in dry methanol (10 ml) was placed in a serum-capped centrifuge tube and deoxygenated with argon. Activated zinc powder (0.5 g) was added, and after further argon flushing, black cobalamin(I) was produced. Neopentyl iodide (50 µl, 75 mg, 0.38 mmol)

was added, the solution became orange-brown and was protected from light.

After 2 min, the tube was centrifuged to remove the unreacted zinc. The supernatant was poured into 1 M HCl (50 ml), and was extracted into phenol : dichloromethane (100 g in 100 ml, 5 ml portions, until the phases were colourless). The combined organic phases were washed with water (20 ml). The dichloromethane was removed by the passage of nitrogen, then the cobalamin was precipitated by the addition of acetone : ether (1 : 9). It was collected by centrifugation and washed four times with dry ether, followed by drying in a stream of dry nitrogen. Yield of neopentylcobalamin was 90 mg (72 %) t.l.c. (cellulose, solvent B) R_{CN} = 1.2 (yellow)

3.5.4 Method C. Cyclooctylcobalamin

Hydroxocobalamin (25 mg, 0.02 mmol) in glacial acetic acid (5 ml) was placed in a centrifuge tube with zinc powder (100 mg). The tube was sealed with a serum cap and flushed with argon. Blackish-green cobalamin(I) was produced within a few minutes. Cyclooctyl iodide (50 mg, 0.17 mmol) in methanol (1 ml) was added through the seal into the foil-wrapped tube. The zinc was removed by centrifugation and the supernatant poured into dry ether (40 ml). The precipitated yellow-orange cobalamin was collected by centrifugation. Yield 11 mg (44 %) contaminated with hydroxocobalamin.

3.6 Extraction through phenol

Solutions of cobalamins were freed from salts by Dolphin's[156] method of extraction through phenol which is summarised below.

The cobalamin solution was extracted with portions of phenol : dichloromethane (100 g in 100 ml). The portions were $^1/5$ of the volume of the aqueous layer, and the extraction was repeated until the phases were colourless. The combined organic extracts were washed with distilled water (2 x $^1/3$ of volume of organic layer), then diluted with dichloromethane to ten times the original volume. The cobalamin was reextracted into portions of distilled water ($^1/20$ volume of the organic layer) until the phases were colourless. The combined aqueous extracts were washed with dichloromethane (3 x volume of aqueous layer) to remove traces of phenol.

The aqueous cobalamin solution was freeze dried, or evaporated on a rotovap (< 40 °).

3.7 Chromatography of cobalamins

Solvent proportions are always quoted by volume.

3.7.1 Thin layer chromatography

Thin layer chromatography on cellulose plates (Merck Art. 5552) was used for the rapid analysis of alkylcobalamins. For cobalamins that are stable in alkaline solution, the solvent system developed by Firth *et al.*[174], for use in paper chromatography, was found to give the best separation and the least streaking. It is referred to as solvent A and is composed of 2-butanol : water : 880 NH_3 (9.5 : 4 : 0.7). The ammonia is present to ensure that the cobalamins remain in the 'base-on' form.

This system sometimes gave rise to streaking, particularly of hydroxocobalamin. In order to see clearly whether hydroxocobalamin was present in a mixture, silica gel plates could be used (Kieselgel 60 F_{254} - Merck Art. 5554). These were eluted with n-propanol : water : 880 NH_3 (100 : 99 : 1). Although the R_f values of alkylcobalamins

were all very similar (*ca* 0.6), the R_f of hydroxocobalamin was $\sim$ 0, which allowed it to be seen very easily as a small compact red spot.

Cobalamins which are unstable in basic solution present more difficulties. Eluents based on mineral acids do not separate alkylcobalamins from desalkyl species. Eluents containing acetic acid are more successful, but cause quite a lot of streaking of spots. The best solvent that was found (solvent B) was 2-butanol : acetic acid : water (10 : 3 : 7).

The R_{CN} value is usually quoted for cobalamins, that is

$$R_{CN} = \frac{\text{distance moved by alkylcobalamin}}{\text{distance moved by cyanocobalamin}}$$, as it is less dependent than the R_f value on slight differences in solvent composition and external conditions. Therefore, standards of cyano- and hydroxocobalamin are run on every plate.

3.7.2 Short column chromatography

The best method for removing traces of hydroxocobalamin from alkylcobalamin preparations was found to be short column chromatography[220] on t.l.c. grade silica gel (Kieselgel 60 H, Merck Art. 7736).

A slurry of silica gel (5 g per 150 mg cobalamin) in the eluting solvent, was poured in to a sinter funnel (internal diameter 2 cm for 100 - 250 mg cobalamin). The cobalamin was applied in solution in the eluting solvent, which was n-propanol : water : 880 NH_3 (100 : 99 : 1). Hydroxocobalamin remained at the top of the column, while alkyl cobalamins were eluted. The solvent was removed by freeze drying.

3.7.3 Ion-exchange chromatography

Short-column chromatography was not suitable for separating a

mixture of alkylcobalamins, although it was very convenient for removing hydroxocobalamin. Therefore, if a mixture of alkylcobalamins had to be separated, ion-exchange carboxymethylcellulose (Whatman CM52 microgranular) was used. The column was prepared in the usual way[173], by first removing fine particles from the cellulose (10 g per 100 mg cobalamin) by washing with water and decanting several times. It was then poured into the column and converted into the H^+ form by the passage of 0.1 M HCl (20 ml) followed by water until the outflow was neutral.

Alkylcobalamins were eluted with water. Hydroxocobalamin could be eluted with 0.1 M HCl.

The disadvantage of this type of column was that it was slow-running, and tended to become slower as the cellulose became more compacted. Also the cobalamins were eluted in large volumes of water as the resolution was not very good. This problem might be overcome by using an eluent of higher ionic strength. A solution of a volatile salt, such as pyridinium acetate, could be used. This could be removed from the cobalamin by evaporation.

Table 3.3: Synthesis of alkylcobalamins

Alkylating agent	Cobalamin	Method	Scale (mg)	Yield (%)	R_{CN}[c,e]
Iodomethane	MeCbl	A(i)	250	90	1.3
Iodoethane	EtCbl	A(i)	100	40	1.4
[2-$^{2}H_3$]Iodoethane	[2-$^{2}H_3$]EtCbl	A(i)	100	70	1.4
[1-$^{2}H_2$]Ethanol *O*-tosylate	[1-$^{2}H_2$]EtCbl	A(i)	90	63	1.4
1-Bromopropane	n-propylCbl	A(i)	50	78	1.6
1-Bromo-n-octane	n-octylCbl	A(i)	100	50	1.6
n-Hexanol *O*-triflate	n-hexylCbl	A(i)	20	75	1.6
Iodocycloatane	cyclooctylCbl	C	50	40	-[b]
Cyclooctanol *O*-triflate	cyclooctylCbl	C	25	0	-[b]
1-Iodo-2,2-dimethylpropane	neopentylCbl	B	125	80	1.1[d]
(*RS*)-Methyloxirane	(*R*+*S*)-2-OHpropylCbl	A(i)	100	52	1.2
(*R*)-Methyloxirane[a]	(*R*)-2-OHpropylCbl	A(i)	200	90	1.2
(*S*)-Methyloxirane[a]	(*S*)-2-OHpropylCbl	A(i)	250	74	1.2
RS-(46)	(*R*+*S*)-2-OH-3,3-dimethylbutylCBl	A(i)	50	88	1.5
RS-(45)	(*R*+*S*)-2-OH-2-cyclohexylethylCbl	A(i)		-[b]	1.4
(*RS*)-Hydroxymethyloxirane	(*R*+*S*)-2,3-diOHpropylCbl	A(i)	100	-[b]	0.8
R-(47)	(*R*)-2,3-diOHpropylCbl	A(i)	100	61	0.7
S-(47)	(*S*)-2,3-diOHpropylCbl	A(i)	250	80	0.7
RS-(21)	(*R*+*S*)-3,4-diOHbutylCbl	A(ii)	250	66	0.9

Table 3.3: contd.

Alkylating agent	Cobalamin	Method	Scale (mg)	Yield (%)	R_{CN} [c,e]
S-(21)	(*S*)-3,4-diOHbutylCbl	A(ii)	100	38	0.9
RS-(24)	(*R*+*S*)-4,5-diOHpentylCbl	A(ii)	200	80	0.9
S-(24)	(*S*)-4,5-diOHpentylCbl	A(ii)	250	76	0.9
(28)[f]	(*R*+*S*)-4,5-diOH-2,2-dimethylpentylCbl	B	100	? 20	1.0[d]

a. Prepared by M.K. Ellis

b. not determined

c. T.l.c. (cellulose, solvent A) unless otherwise specified

d. T.l.c. (cellulose, solvent B)

e. $R_{CN} = R_f(XCbl)/R_f(CNCbl)$

f. contaminated with (41)

CHAPTER 4

SPECTROSCOPY OF ALKYLCOBALAMINS

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SPECTROSCOPY OF ALKYLCOBALAMINS

4.1 Electronic Spectra

Vitamin B_{12} and its derivatives are all intensely coloured, and this colour can vary according to the oxidation state of the cobalt atom, the pH of the solution, and the nature of the axial ligands. The ultraviolet-visible spectra of corrinoids are correspondingly sensitive to these factors, and so these spectra give information as to the behaviour and structure of corrinoids in solution.

The spectra of most cobalamins contain three main absorption bands, α,β and γ, in addition to some weaker DE bands and transitions at higher energy, δ (Figure 4.1). Firth *et al.*[175] collected the UV visible spectra of a large number of cobalamins and cobinamides, as well as information about the circular dichroism of these compounds, and they and others have attempted to assign the bands to specific transitions and to study how the wavelengths and extinction coefficients vary with changes in the axial ligands. The assignment of bands was aided by the isolation by Toohey[176] of an orange, cobalt-free, corrinoid from *Chromatium*. This compound was shown to be metal-free by X-ray fluorescence and emission spectrum analyses, and was found to take up cobalt under certain conditions to give an absorption spectrum identical with that of cobamide. Its structure was thought to be corrin-aminopropanol-phospho-ribose, which was later confirmed[177]. It can be named hydrogenobamide, since a proton replaces the cobalt in cobamide. The ultraviolet-visible spectrum of this compound (Figure 4.2) shows the same main bands as the spectrum of cobalt-

Figure 4.1 UV-visible spectrum of $(CN)_2Cbl$.

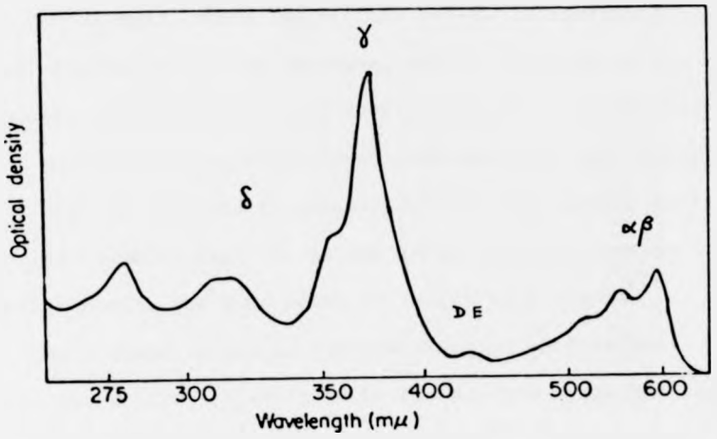
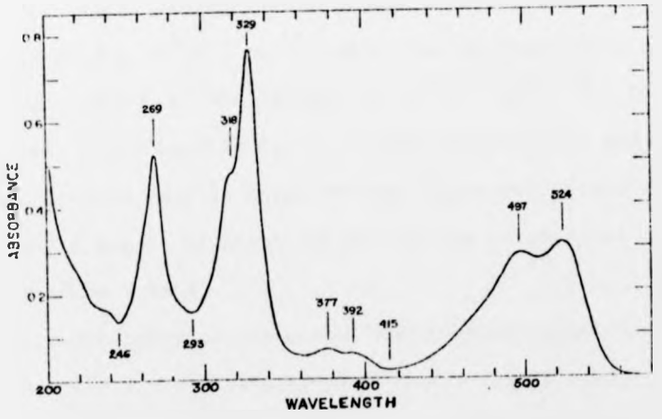


Figure 4.2 UV-visible spectrum of cobalt-free corrinoid.



containing corrinoids, indicating that these bands are due to $\pi \rightarrow \pi^*$ transitions of the corrin chromophore, and not to any transitions involving the cobalt atom.

The $\alpha\beta$ band, which imparts the colour to corrinoids, is at the lowest energy end of the spectrum, and is assigned to the HOMO-LUMO (symmetry forbidden $\pi_7 \rightarrow \pi_8^*$) transition of the chromophore[177]. The α and β bands are vibrational components of this transition. The energy of the band in general follows the nephelauxetic series of axial ligands, that is, as the axial ligands increase in electron-donating power, the band moves to longer wavelengths[175].

The γ band, which is the most intense band in most cobalamin spectra, is assigned to the allowed symmetric combination of the $\pi_7 \rightarrow \pi_9^*$ and $\pi_6 \rightarrow \pi_8^*$ configurations[177]. It also follows the nephelauxetic series, moving to longer wavelengths as the total electron-donating power of the axial ligands increases. It decreases in intensity as the difference in donor strengths of the axial ligands increases. Thus, dicyanocobalamin has a very intense γ band at 367 nm (ε 30.8 x 10^3 M^{-1} cm^{-1}), while the corresponding band in hydroxocobalamin is at 358 nm (20.6 x 10^3 M^{-1} cm^{-1})[178]. In organo-cobalamins, the γ band is hardly visible between 350 and 400 nm, and the most intense band is below 300 nm, sometimes called γ_2[175]. The weak D and E bands, at about 420 nm, may be vibrational components of the intense γ band.

There are other possible electronic transitions of cobalamins, other than the $\pi \rightarrow \pi^*$ transitions of the corrin ligand. They are i) d-d transitions of the cobalt ion, ii) internal transitions of the axial ligands, iii) charge transfer from the axial ligand to cobalt, or *vice versa*, and iv) charge transfer from the corrin to cobalt, or *vice versa*.

The d-d transitions of the octahedrally coordinated cobalt(III) ion are symmetry-forbidden, and so will be of low intensity. They are therefore likely to be hidden beneath other more intense absorptions.

Charge transfer bands are likely to be of quite high intensity, with wavelengths depending on the oxidation state of the cobalt and the nature of the ligands[177]. One such band has been unambiguously assigned[178] in the case of cobalamins. The spectrum of phenolato-cobalamin when compared with that of hydroxocobalamin shows an additional intense broad band at 455 nm, assigned to be a charge transfer band from the phenolate anion to cobalt.

Alkyl-cobalt complexes show no such obvious band, but studies on the photolysis of methylcobalamin[179] and methylcobaloxime[180], suggest that charge transfer bands are hidden beneath the $\pi \rightarrow \pi^*$ transitions in the visible region of the spectrum, and excitation of these frequencies causes homolysis of the Co-C bond. Other authors[181] interpret the results to show that excitation of the $\pi \rightarrow \pi^*$ transitions of the corrin chromophore in this region (the α β band) causes homolysis.

In acid solution, alkylcobalamins change colour from red to yellow with pKa 0.7 - 4.8, and show the same UV-visible spectra as the corresponding cobinamides. This shows that the 5,6-dimethyl-benzimidazole base is protonated and so not coordinated to the cobalt atom. In compounds such as cyano- and aquocobalamin the axial base is much more strongly coordinated, so a much lower pH is needed to give the 'base-off' form.

The UV-visible spectra are useful for distinguishing alkyl-cobalamins from ones with inorganic ligands, and for assessing the electrochemical and pH-dependent behaviour of cobalamins. Reactions

of cobalamins can be followed spectroscopically, and sometimes the UV-visible spectrum is the only way of characterising a cobalamin, or other organo-cobalt derivative, particularly if it is too unstable to be isolated or chromatographed[182].

The molar extinction coefficients of cobalamins cannot be measured directly, because of the tendency of cobalamin crystals to contain large and variable amounts of water of crystallisation, which gives an unreliable estimate of the molecular weight. All cobalamins, however, decompose to give the violet-coloured dicyanocobalamin (56) in the presence of light and excess cyanide solution, and so this compound can be used as a standard. The molar extinction coefficient of its γ band is accurately known (30.8×10^3 M^{-1} cm^{-1} at 367 nm)[183], and so the concentration of a solution of an alkylcobalamin can be calculated by converting it into dicyanocobalamin.

The UV-visible spectra are not so useful for determining precise structural features of alkylcobalamins, since most have broadly similar spectra, and the differences in absorption maxima are not easy to interpret. The purity of an alkylcobalamin is also difficult to assess from the absorption spectrum, as a high proportion of desalkylcobalamin can be hidden under the broad bands of the alkylcobalamin spectrum.

In the present work, UV-visible spectroscopy was not used to any great extent. Other techniques were found to be more useful for determining the purity and investigating the structure of alkylcobalamins. The absorption spectra of methyl- and ethylcobalamin and (*R*)- and (*S*)-dihydroxypropylcobalamin were recorded. The diastereomeric cobalamins had practically identical spectra (Figure 4.3) as did the other alkylcobalamins (Table 4.1). This technique is therefore not sensitive to small differences in the alkyl ligand.

The extinction coefficients of these compounds were calculated

Table 4.1: UV-visible data of cobalamins

MeCbl		EtCbl		Dihydroxypropyl Cbl (*R*)		Dihydroxypropyl Cbl (*S*)		OHCbl		$(CN)_2$Cbl	
λ	($\varepsilon \times 10^{-4}$)	λ	($\varepsilon \times 10^{-4}$)	λ	($\varepsilon \times 10^{-4}$)	λ	($\varepsilon \times 10^{-4}$)	λ	($\varepsilon \times 10^{-4}$)	λ	($\varepsilon \times 10^{-4}$)
266	(2.02)	264	(2.00)	265	(2.07)	263	(2.10)	272	(2.03)	277	(1.75)
279	(1.90)	278	(1.82)	282	(1.84)	281	(1.93)			288	(1.29)
290	(1.79)	290	(1.74)	288	(1.78)	287	(1.83)				
316	(1.34)	306	(1.51)	318	(1.36)	316	(1.24)			311	(1.11)
341	(1.43)	346	(1.33)	340	(1.28)	338	(1.30)	340	(2.53)	368	(3.09)
374	(1.19)			375	(1.04)	376	(1.04)	411	(0.38)	417	(0.27)
522	(0.94)	509	(0.89)	515	(0.85)	522	(0.84)	523	(0.90)	540	(0.90)
										579	(1.04)

λ = absorption maximum (nm)

ε = molar extinction coefficient (M^{-1} cm^{-1})

Concentrations ranged from 1.3 - 2.7 x 10^{-5} M.

by the conversion of the alkylcobalamin into dicyanocobalamin, which has a well defined molar extinction coefficient (see above). This was achieved by the addition of a crystal of KCN to the cell containing the cobalamin solution, followed by exposure of the solution to light. Conversion into dicyanocobalamin was then complete within a few hours (Scheme 4.1).

Scheme 4.1

$$RCbl \xrightarrow{light} OHCbl \xrightarrow{CN^-} (CN)_2Cbl$$

4.2 Infrared spectroscopy

The infrared spectroscopy of cobaloximes was studied by Yamazaki and Hohokabe[184], who assigned most of the bands in a wide range of cobaloxime spectra. They found the Co-C stretching vibration in alkylcobaloximes to be at about 325 cm^{-1}, a result which was confirmed by other workers[185] who assigned peaks at 332 and 320 cm^{-1} in alkylcobaloxime spectra to the symmetric and asymmetric stretching modes of the Co-C bond.

Examples of the infrared spectra of hydroxo-, methyl-, adenosyl- and cyanocobalamin are given by Hogenkamp *et al.*[153]. While these compounds have very different UV-visible spectra, their IR spectra are almost indistinguishable. The cobalt-carbon bond should absorb between 300 and 450 cm^{-1}, but all cobalamins, whether or not they contain a cobalt-carbon bond, have a strong broad absorption between 350 and 600 cm^{-1}, possibly due to Co-N stretching modes. Only methylcobalamin shows a weak resonance at 348 cm^{-1}, probably corresponding to a Co-C stretching vibration. As expected, this peak diminishes in intensity upon irradiation of the sample.

In the present work it was of interest to see whether the spectra of specifically deuterated alkylcobalamins could be distinguished by IR spectroscopy, as this would open up the possibility of following

reactions of alkylcobalamins in solution, and also of determining the fates of specific hydrogen atoms on the alkyl ligand. This would be useful in vitamin B_{12} model reactions, where intramolecular hydrogen atom transfer is being investigated.

The difference in mass between a deuteron and a proton means that the C-D stretching frequency should be $^{1}/1.37$ of the corresponding C-H frequency[186]. For an aliphatic C-D, this should place the absorption between 2000 and 2200 cm^{-1}, in a region free from other bands, even if it is of very low intensity. It is likely to be of low intensity compared to the C-H stretching band in a cobalamin labelled on the alkyl ligand, as there would be so many more C-H bonds than C-D bonds in the molecule.

Deuterated ethylcobalamins were chosen as simple model compounds in order to try to assign the C-D stretching frequencies. [1,1-$^{2}H_{2}$]- and [2,2,2-$^{2}H_{3}$]-ethylcobalamin were prepared from [1,1-$^{2}H_{2}$]-ethanol-*O*-tosylate and 2,2,2-[$^{2}H_{3}$]-iodoethane, respectively. The alkylating agents contained < 2 % incompletely labelled species, and the ^{1}H NMR spectra (400 MHz) of the ethylcobalamins gave the expected signals.

The IR spectra were recorded initially on an ordinary IR spectrometer (Perkin-Elmer ^{257}), as nujol mulls with NaCl plates, and no differences were detected between the spectra of the samples. Spectra recorded on a Nicolet FT-IR spectrometer also did not show any significant differences, either in solution (H_2O, CaF_2 cell) or in nujol mull, even though up to 12000 scans were accumulated in the hope of detecting very low intensity absorptions. The Co-C band was not observed because the plates were not transparent down to 300 cm^{-1}. The ethylcobalamins did not decompose during the experiments, as shown by the t.l.c. of the recovered samples.

The C-D absorption bands might be shifted to an unexpected frequency, and lowered in intensity, by the proximity of the cobalt atom, and so they may be hidden by other resonances. Further investigation of this problem is required.

4.3 The nuclear magnetic resonance spectroscopy of cobalamins

4.3.1 Introduction

Nuclear magnetic resonance spectroscopy (NMR) has proved to be a useful technique for the investigation and analysis of alkylcobalamins. While other techniques such as ultraviolet-visible and infrared spectroscopy (sections 4.1 and 4.2) give some information about the cobalamin, the spectra of alkylcobalamins all tend to be similar, and so give little information about the structure and purity of specific samples.

It was found that by using high field proton NMR, the β-ligands of alkylcobalamins could often be seen, and so the identification of alkylcobalamins was put on a much firmer basis than when the only methods of analysis were UV-visible spectroscopy and t.l.c. It was also found to be possible to distinguish the diastereoisomers of cobalamins synthesised from pairs of enantiomeric alkylating agents, which was not possible by any other physico-chemical technique (except, of course, X-ray crystallography, which is not practicable as a routine method of analysis).

A disadvantage of NMR as a spectroscopic technique is its intrinsically low sensitivity, which means that relatively large samples are required, even for proton NMR, which gives the greatest

sensitivity. In the present work, *ca.* 10 mM solutions of cobalamins in ^2H_2O or [$^2H_4$]-methanol were used, that is, *ca.* 7 mg in 0.5 ml solvent. This allowed spectra with acceptable signal to noise ratios to be obtained at 400 MHz, without the need for very long accumulation times. With solutions of this strength, however, intermolecular interactions may affect the structure of the cobalamin molecules.

High resolution NMR spectra can only be obtained with diamagnetic compounds. Traces of paramagnetic compounds cause serious line-broadening, because of more efficient relaxation mechanisms. This is not in general a problem for alkylcobalamins, where the cobalt(III) species are diamagnetic, but the photolysis and thermolysis of alkylcobalamins, produced solutions containing cobalt(II) species, and giving unresolved spectra. Oxygen had to be passed through the solution to reoxidise the cobalt(II) to cobalt(III), before the spectrum could be recorded. This resulted in loss of information about the initial cobalt-containing products of the reactions.

In order to identify the resonances arising from the alkyl ligand of a particular cobalamin, to characterise the compound, it was necessary to be sure of the assignments of the resonances from the rest of the cobalamin. Two main studies of the high field proton NMR spectra of vitamin B_{12} derivatives have been made, by Hill and co-workers[187] and by Battersby *et al.*[188]. The first study[187] (we are grateful to the authors for a pre-publication copy of this reference) was an investigation of a number of cobalamins, including the coenzyme, adenosylcobalamin. Using a combination of decoupling techniques, various shift reagents, and pH titrations, the authors assigned the resonances of all the protons in the methyl groups, the 5,6-dimethylbenzimidazole, the ribose and the propanolamine

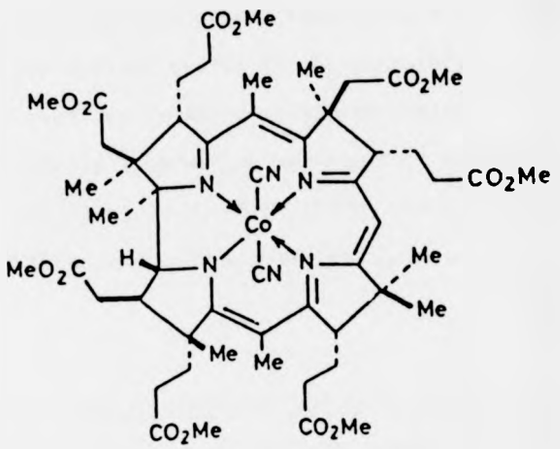
group. They also assigned the resonances of the β-ligand in methyl-, ethyl- and adenosylcobalamin.

Using these results as a starting point, a detailed study of a number of alkylcobalamins was made in the present work, starting with methylcobalamin. Most of the assignments made by Hill *et al.*[187] were confirmed by independent methods, including n.O.e. difference spectroscopy. In addition, the resonances of the five methine protons on the corrin ring were identified. Four of these project towards the β-face of the corrin, and so are likely to be most affected by differences in the β-ligand. It was therefore important to be sure of their assignments.

The methylene protons of the propionamide and acetamide side chains were not assigned, as they gave rise to a largely unresolved 'methylene envelope'.

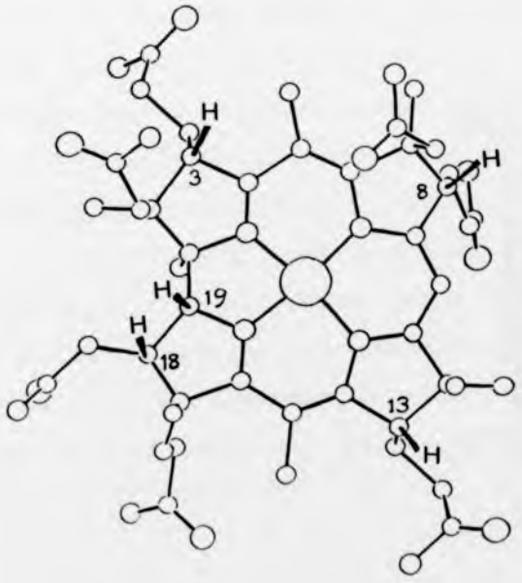
This did not deter the authors of the later study[188], which was published during the present work, who assigned every resonance in the 400 MHz ^{1}H NMR spectrum of dicyanoheptamethylcobyrinate (cobester) (57) in [$^{2}H_{6}$]-benzene. Extensive use was made of n.O.e. difference and decoupling difference spectroscopy, as well as relaxation time studies. Using selective {^{1}H}-decoupling, and specifically deuterated cobester, the ^{13}C-spectrum was also assigned.

Although the solvent was different from those used in the present work ([$^{2}H_{6}$]-benzene rather than $^{2}H_{2}O$ or [$^{2}H_{4}$]-methanol), and cobester lacks the 5,6-dimethylbenzimidazole base, and associated ribose, phosphate and propanolamine moieties, the remaining resonances showed a qualitative agreement with those of cobalamins. For the methyl groups, only Cl-CH_{3} showed a large difference (*ca.* 1 p.p.m. downfield in the cobester) which was probably due to the removal of the shielding effect of the 5,6-dimethylbenzimidazole base. The



(57) Cobester

Figure 4.11 Cobalamin showing positions of methine protons on β-face.



other methyl groups projecting towards the α-side of the corrin showed slight upfield shifts in the cobester, probably because in cobalamins they are in the deshielding region of the aromatic base.

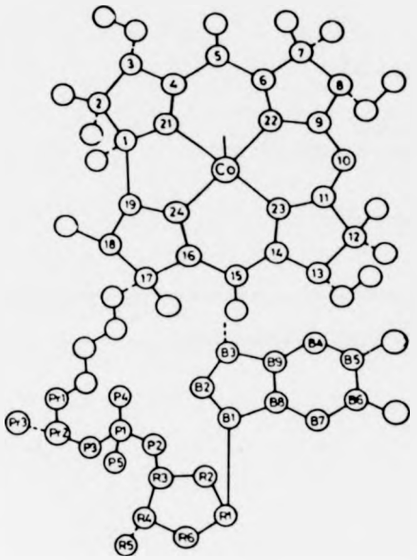
Particularly interesting was the fact that the methine protons on the corrin ring, C3-H, C8-H, C13-H, C18-H and C19-H, had chemical shifts in the same order as those we assigned in methylcobalamin.

4.3.2 The spectra of alkylcobalamins and their assignment

The ^{1}H NMR spectra of alkylcobalamins show several distinct regions, and most of the resonances show only slight (although not insignificant) changes on alteration of the alkyl ligand (see Table 4.2). The signals are quite broad, probably as a result of the slow tumbling rate of cobalamins in aqueous or methanolic solution, which decreases the transverse relaxation time T_2, and so increases the linewidth. These solvents were used because cobalamins are insufficiently soluble in less polar solvents. Alternatively, this effect may be produced because of traces of Co(II) species in the solution, as paramagnetic ions increase the linewidth, but this is less likely, since resonances from small molecules in the cobalamin solution were not broadened to the same extent.

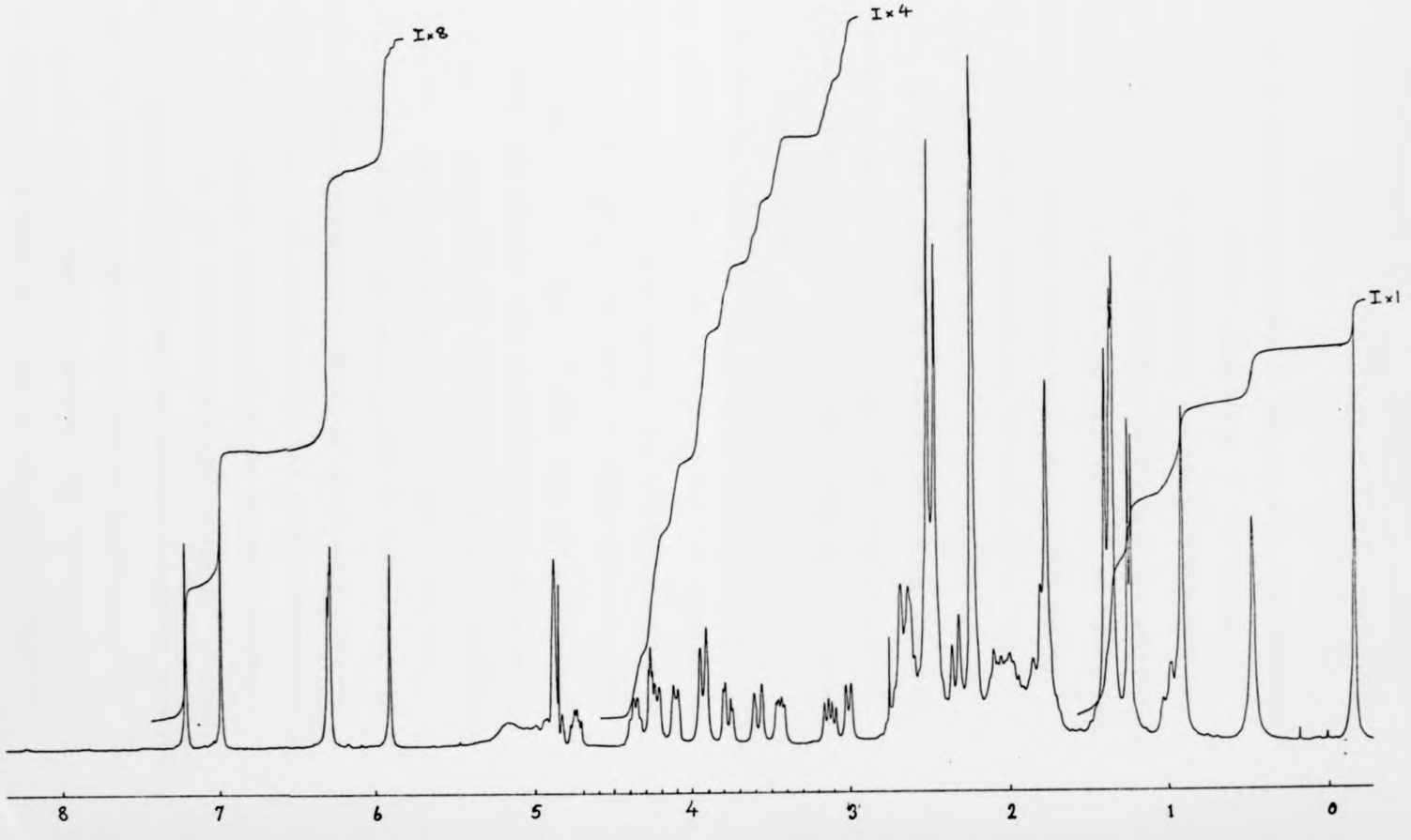
The spectrum of methylcobalamin in D_2O is described below. In general, spectra were run with automatic suppression of the residual solvent peak. The numbering system is shown in figure 4.5 following the IUPAC convention. The spectrum of methylcobalamin (300 MHz, $^{2}H_2O$) is shown in figure 4.6.

Figure 4.5: IUPAC numbering system of cobalamin



1) The region downfield of the HOD signal contains five signals, of which all but one are singlets. The only doublet is therefore assigned to be R1-H on the ribose ring. The four singlets include the aromatic protons on the 5,6-dimethylbenzimidazole base, and C10-H on the corrin. These were assigned by Hill *et al.*[187] as follows; C10-H exchanges readily in acid solution, and its resonance moves downfield when the cobalamin becomes base-off, in acid solution. It was therefore assigned as the signal at δ 5.90. Of the other three resonances, only one (δ 6.97) exchanged[189] in $^{2}H_{2}O$ at 95 °, showing it to be B2-H. B4-H and B7-H were assigned by their different paramagnetic shifts on binding of a lanthanide shift reagent to the phosphate diester, and comparison with the crystal structure.

Figure 4.6 ^{1}H NMR spectrum of methylcobalamin (300 MHz, D_2O)



2) The region immediately upfield of HOD (δ 4.6 - 2.8) contains twelve single-proton resonances, comprising all the methine and methylene protons in the propanolamine and ribose portions of the molecule, and four of the five methine resonances on the corrin ring. C3-H, C8-H, C13-H and C19-H. Of these Hill *et al.*[187] had assigned the ribose and propanolamine protons by sequential decoupling, starting with the already assigned R1-H (δ 6.28, d), and the propanolamine methyl group (δ 1.22, d). We confirmed these assignments for methylcobalamin by a similar decoupling sequence.

We assigned the remaining resonances at δ 3.01, 3.40, 3.93, 4.17 as C13-H, C8-H, C19-H and C3-H, respectively, by difference n.O.e. experiments (section 4.3.3c).

3) The highfield region of the spectrum (δ 0 - 2.5) contains ten singlets corresponding to methyl groups, one doublet, assigned to the Pr1-CH_3, and an unresolved envelope of signals from δ 0.7 to 2.5, corresponding to the methylene signals from the acetamide and propionamide side chains. The resonance of the remaining methine proton on the corrin ring (C18-H), was shown by irradiation of C19-H to be at about δ 2.58, and an n.O.e. when C1-CH_3 was irradiated confirmed this. The methyl groups of the coenzyme had been assigned[187] by observing the effects of metal ion binding to the phosphate diester, and comparing with the crystal structure. The interatomic distances calculated from the crystal structure were in agreement with the relative magnitudes of the Pr^{3+}-induced paramagnetic shifts, suggesting that the conformation of the coenzyme in solution is not very different from that in the crystal.

One methyl group appeared at unusually high field, δ 0.47. This was assigned to C1-CH_3, as it lies in the shielded region of

the aromatic 5,6-dimethylbenzimidazole base. Base-off cobalamins show a shift of this resonance to higher field.

4) In addition, the spectrum of methylcobalamin contains a broad singlet at δ-0.12. This corresponds to the Co-CH_3 group, shielded either by electron-donation from the cobalt atom, or because of its position relative to the conjugated corrin system. The appearance of signals at high field is characteristic of the protons on the alkyl ligand of cobalamins. Such shielding is not seen, for instance, in alkylcobaloximes[56], where the highest field resonances are at *ca.* δ 0.6. Therefore the shielding must be largely due to the corrin system rather than to the cobalt atom. This is confirmed by the fact that in cobalamins with protons on C1 and C2 of the alkyl ligand, the protons on C2 are shifted upfield more than those on C1, since although they are further from the cobalt atom, they are presumably nearer to the conjugated system. Protons on the third or subsequent carbon atoms are shielded to a lesser extent, and approach the usual chemical shifts for such protons in normal aliphatic compounds.

Spectra of other alkylcobalamins were assigned either by direct comparison with that of methylcobalamin, and by a combination of decoupling and decoupling difference spectroscopy to assign the resonances of the various alkyl ligands (section 4.3.4). In two other cases, the assignments of C3-H, C8-H, C13-H and C19-H were confirmed by n.O.e. difference spectroscopy.

The chemical shifts of a number of cobalamins assigned in this work are listed in Table 4.2. The chemical shifts and coupling constants of selected protons are listed in Table 4.3.

Table 4.2: Chemical shifts for certain alkylcobalamins

			OHpropyl		diOHpropyl			diOHbutyl	
Solvent	Methyl D_2O	Ethyl D_2O	*R*- D_2O	*S*- D_2O	*R*- D_2O	*S*- D_2O	*S*- CD_3OD	*R*- CD_3OD	*S*- CD_3OD
B7	7.19	7.16	7.17	7.17	7.18	7.18	7.20	7.14	7.14
B2	6.97	7.02	7.00	6.97	6.99	6.97	7.18	7.10	7.10
B4	6.27	6.28	6.26	6.30	6.26	6.28	6.48	6.38	6.39
R1	6.28	6.24	6.26	6.26	6.26	6.27	6.23	6.18	6.18
C10	5.90	6.07	6.07	6.03	6.04	6.02	6.14	6.09	6.09
R3	4.71	∿4.7	∿4.7	∿4.7	∿4.7	∿4.7	4.75	4.69	4.69
Pr2	4.33	4.34	4.34	4.32	4.34	4.34	4.39	4.36	4.36
R2	4.23	4.26	4.25	4.23	4.25	4.25	4.25	4.21	4.21
C3	4.17	∿4.05	4.08	4.07	4.05	4.04	4.31	4.23	4.22
C19	3.94	4.14	4.57	4.21	4.60	4.17	4.37	4.17	4.17
R4	4.08	∿4.12	4.11	4.21	4.10	4.10	4.17	4.13	4.13
R5α	3.90	3.89	3.89	3.89	3.90	3.90	3.91	3.89	3.89
R5β	3.74	3.75	3.75	3.74	3.76	3.76	3.78	3.74	3.74
Pr1α	3.56	3.53	3.53	3.55	3.53	3.54	3.64	3.58	3.58
C8	3.40	3.40	3.43	∿3.2	3.40	3.26	3.55	3.63	3.61
C13	3.01	3.20	3.22	∿3.2	3.22	3.22	3.30	3.24	3.25
Pr1β	3.11	3.14	3.16	3.12	3.15	3.13	3.05	∿3.0	∿3.0
C18-H	∿2.7	-				∿2.6		∿2.8	2.81
C5	2.48	2.50	2.51	2.50	2.51	2.51	2.58	2.55	2.55
C15	2.45	2.48	2.49	2.50	2.50	2.51	2.56	2.52	2.53
B5	2.21	2.21	2.22	2.22	2.22	2.22	2.30	2.26	2.26
B6	2.21	2.21	2.22	2.22	2.22	2.22	2.29	2.25	2.25
C7	1.76	1.79	1.79	1.87	1.79	1.85	1.89	1.83	1.83
C12α	1.39	1.45	1.43	1.41	1.44	1.41	1.46	1.48	1.48
C2	1.34	1.39	1.43	1.39	1.43	1.36	1.38	1.39	1.39
C17	1.34	1.39	1.39	1.35	1.39	1.38	1.42	1.39	1.38
Pr3	1.22	1.20	1.15	1.23	1.21	1.21	1.25	1.22	1.22
C12β	0.91	1.07	1.20	1.20	1.16	1.24	1.27	1.11	1.12
C1	0.47	0.51	0.50	0.40	0.50	0.42	0.48	0.53	0.52

Table 4.2: contd.

Solvent	diOHpentyl *R*- D_2O	diOHpentyl *S*- D_2O	*S*- CD_3OD	diOH hexyl *(R+S)*-	major isomer 2-t-butyl-2-OH-ethyl D_2O/CD_3OD	D_2O 2-cyclohexyl 2-OHethyl major	D_2O 2-cyclohexyl 2-OHethyl minor	Adenosyl D_2O
B7	7.18	7.18	7.15	∿7.3	7.16	7.27	7.27	7.16
B2	6.98	6.98	7.14	7.22	6.95	6.98	6.94	6.94
B4	6.26	6.26	6.18	6.43	6.86	∿6.27	6.28	6.23
R1	6.26	6.27	6.42	6.29	6.25	∿6.27	6.27	6.26
C10	6.07	6.07	6.13	6.19	5.98	6.04	5.99	5.95
R3	4.73	4.73	4.69		4.69	4.73	4.73	4.72
Pr2	4.34	4.34	4.36		4.31	4.34	4.34	4.33
R2	4.24	4.24	4.23	4.20	4.21	4.25	4.24	4.24
C3	4.09	4.09	4.32	4.34	4.03	∿4.07	∿4.07	4.13
C19	4.15	4.15	4.17		4.69	4.58	4.20	4.23
R4	4.10	4.10	4.15	4.10	∿4.07	∿4.10	∿4.10	∿4.10
R5α	3.90	3.90	3.88	3.89	3.88	3.90	3.90	3.88
R5β	3.74	3.74	3.74	3.75	3.73	3.75	3.75	3.73
Pr1α	3.53	3.53	3.59		3.56	3.54	3.55	3.54
C8	3.41	3.41	3.67	3.15	3.49	3.45	3.24	3.31
C13	?	3.22	3.25	3.75	3.12	∿3.19	∿3.19	2.90
Pr1β	3.17	3.17	3.03		3.08	3.16	3.16	3.12
C18-H	?	∿2.6	2.82					
C5	2.51	2.51	2.55	2.50	2.50	2.50	2.49	2.46
C15	2.49	2.49	2.53	2.48	2.48	2.49	2.49	2.44
B5	2.22	2.22	2.26	2.22	2.22	2.21	2.21	2.19
B6	2.22	2.22	2.25	2.21	2.20	2.21	2.21	2.19
C7	1.79	1.79	1.83	1.80	1.79	1.78	1.85	1.72
C12α	1.46	1.46	1.49	1.48	1.45	1.44	1.45	1.37
C2	1.41	1.41	1.38	1.41	1.36	1.39	1.39	1.35
C17	1.39	1.39	1.38	1.40	1.31	1.32	1.30	1.32
Pr3	1.20	1.19	1.22	1.20	1.21	1.22	1.22	1.22
C12β	1.10	1.10	1.10	1.09	1.28	1.20	1.21	0.86
C1	0.52	0.52	0.54	0.56	0.45	0.50	0.42	0.47

Table 4.2: contd.

Solvent	n-octyl D_2O	neopentyl DCl/D_2O 0.1 M	S-diOHpropyl DCl/KCl pH 4.0
B7	7.16	9.26	7.26
B2	6.98	7.61	6.55 (br)
B4	6.26	7.52	6.25 (br)
R1	6.25	6.59	6.33
C10	6.05	7.03	
R3	∿4.7		
Pr2	4.33	5.02	4.2
R2	4.24	4.91	-
C3	4.14	4.79	4.39
C19	∿4.10	4.79	
R4	∿4.10	∿4.45	4.05
R5α	3.89	4.30	3.89
R5β	3.74	4.10	3.75
Pr1α	3.52	3.5	3.47
C8	3.45	-	3.40
C1B	∿3.17	4.0	3.29
Pr1β	∿3.14	∿3.3	3.19
C18-H		2.87	
C5	2.51	2.41	2.49
C15	2.47	2.34	2.48
B5	2.21	2.24	2.23
B6	2.21	2.24	2.21
C7	1.78	1.87	1.85
C12α	1.45	1.64	1.44
C2	1.38	1.44	1.42
C17	1.38	1.42	1.38
Pr3	1.20	1.24	1.21
C12β	1.07	1.19	1.19
C1	0.51	0.69	0.50

Table 4.3a: Chemical shifts and coupling constants of the ribose and propanolamine groups of certain alkylcobalamins

	Alkyl group								
δ (p.p.m.)	OHpropyl (*R*)	OHpropyl (*S*)	diOHpropyl (*R*)	diOHpropyl (*S*)	diOHbutyl (*R*)*	diOHbutyl (*S*)*	methyl	adenosyl(**)	
Proton									
R1	6.25	6.26	6.26	6.26	6.18	6.18	6.28	6.26	(6.23)
R3	∿4.7	∿4.7	∿4.7	∿4.7	4.67	4.69	4.72	4.72	(4.74)
Pr2	4.34	4.32	4.34	4.33	4.36	4.36	4.32	4.33	(4.32)
R2	4.25	4.23	4.25	4.23	4.21	4.21	4.24	4.24	(4.23)
R4	∿4.11	4.09	4.11	4.09	4.13	4.13	4.08	∿4.10	(4.09)
R5β	3.89	3.89	3.90	3.89	3.89	3.89	3.90	3.88	(3.89)
R5α	3.74	3.74	3.75	3.74	3.74	3.74	3.75	3.73	(3.74)
Pr1α	3.53	3.55	3.54	3.55	3.58	3.58	3.56	3.54	(3.55)
Pr1β	3.16	3.12	3.16	3.23	∿3.00	∿3.00	3.11	3.12	(3.16)
Pr3	1.21	1.20	1.20	1.20	1.21	1.22	1.22	1.22	(1.21)
J(Hz)									
Pr3-Pr2	5.8	6.3	6.3	6.3	6.3		6.2	6.2	(6.0)
Pr2-Pr1α	∿2	∿1	∿0	∿0	∿1		2.5	2.5	(∿ 0)
Pr2-Prβ	7.1	7.6	7.2	∿6	7.9		8.0	7.2	(7.3)
Pr2-P	?	∿8	?	?	∿8		8.0	?	(5.9)
Pr1α-Pr1β	14.5	14.8	14.5	∿14	13.9		14.3	14.0	(13.2)
R1-R2	3.5	3.0	3.4	3.4	3.1		2.6	2-3	(2.4)
R2-R3	3-4	∿3	4.3	4	4.4		4.0	4.0	(3.8)
R3-R4	8.8	∿8	8.5	8.8	8.0		8.4	∿10	(7.4)
R4-R5α	3.8	∿3.5	3.6	3.8	4.0		3.6	2.3	(0)
R4-R5β	2.5	2.3	1.9	2.5	2.0		2.5	2.3	(3.7)
R5α-R5β	12.9	12.8	12.8	13.0	12.5		13.0	12.8	(11.8)
R3-P	?	?	?	?	8.0		8.4	?	(7.4)

* These spectra were obtained in [$^2H_4$]-methanol, the others were in D_2O.

** Values in brackets are taken from ref. 187.

Table 4.3 : Methine from selected cobalamins

chemical shift (δ, p.p.m.) (coupling constant (J, Hz))

Alkyl ligand		C3-H	C8-H	C13-H	C19-H
methyl		4.17 (9.1, 2.3)	3.40 (10.9, 5.0)	3.02 (11.2, ∿1)	3.94 (9.7)
ethyl		4.05 (8.7)	3.40 (11.0, 4.2)	3.2 (> 8)	4.14 (> 10)
OHpropyl (*R*)		4.08 (8.8, ∿ 2)	3.43 (11.3, 5.0)	3.22 (9.3)	4.57 (10.5)
(*S*)		4.07 (9.2)	∿3.2 (∿ 11, ∿ 6)	∿3.2 (> 9)	4.21 (> 10)
diOHpropyl (*R*)		4.05 (9.1)	3.40 (11.1, 4.8)	3.22 (9.4)	4.60 (10.6)
(*S*)		4.04 (9.1)	3.29 (11.1, 5.0)	3.22 (∿ 10)	4.17 (10.4)
diOHbutyl (*R*)*		4.23 (8.7)	3.62 (∿ 10,∿5.5)	3.24 (8.9, 3.3)	4.17 (∿ 11)
(*S*)*		4.22 (8.7)	3.61 (9.9, 5.4)	3.25 (8.9, 3.3)	4.17 (11.3)
diOHpentyl (*R & S*)*		4.09 (?)	3.41 (11.3, 4.9)	3.21 (9.0, 2.8)	4.15 (10.4)
2-t-butyl-2-hydroxy-ethyl	(major)	4.03 (∿ 9)	3.44 (∿ 11, ∿5)	3.19 (∿ 10)	4.69 (∿ 11)
	(minor)	4.03 (∿ 9)	3.25 (?)	∿3.2 (?)	4.17 (∿ 1)
2-cyclohexyl-2-hydroxy-ethyl	(major)	4.07 (∿ 9)	3.45 (11.5, 5)	3.19 (∿ 11)	4.58 (∿ 11)
	(minor)	4.07 (∿ 9)	3.25 (11.5, 5)	3.20 (∿ 11)	4.20 (∿ 11)
adenosyl		4.10 or 4.23	3.31 (11.2, 4.9)	2.90 (9, 1)	4.23 or 4.10

* These spectra were obtained in [$^2H_4$]-methanol

4.3.3 Nuclear Overhauser effect difference spectroscopy

4.3.3.a. The nuclear Overhauser effect

The nuclear Overhauser effect (n.O.e.) is defined as the change in intensity of the signal of one proton or group of protons (B), when another proton or group of protons (A) is saturated with a radiofrequency pulse[190].

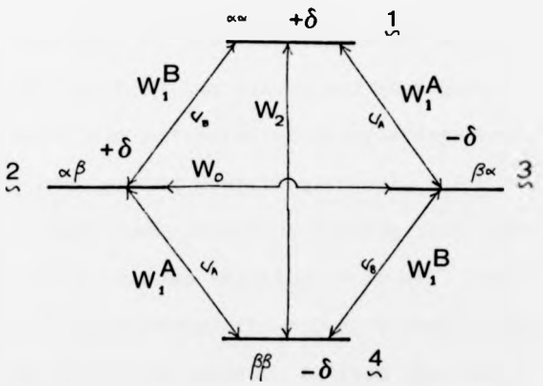
In general, the intensity of signal B depends on the efficiency of relaxation of the excited nuclei back to the equilibrium Boltzmann distribution. In organic molecules in solution, dipole-dipole interactions with neighbouring protons are usually the most important contributors to this relaxation. This is a through space effect, depending on the inverse sixth power of the distance between the interacting protons[191].

On irradiation of a nucleus A, which contributes to the relaxation of B, the efficiency of this dipolar cross-relaxation is increased, and the signal B is altered. The magnitude of the effect therefore also depends on $(r_{AB})^{-6}$, so a comparison of the n.O.e.s of various protons gives an indication of their spatial relationships. Strictly speaking, the $(r_{AB})^{-6}$ relationship assumes that all the protons have the same longitudinal relaxation time T_1. In organic molecules, only protons of the same type have similar T_1 values, that is, in general, methylene protons will have a different relaxation rate from methyl protons. Therefore only protons of the same type can be compared directly. A proton is efficiently relaxed by a *geminal* partner which makes the contribution of other nuclei to the total relaxation less important, so, for instance, the relative n.O.e. of a methyl group will be smaller than that of an isolated methine proton.

For small organic molecules the n.O.e. is usually positive, that is, the signal of B is enhanced, but for large molecules it can be negative. This is explained by the use of a Solomon diagram, showing schematically a two spin system AB (Figure 4.7). The transition probabilities, W, correspond to the ways in which an excited nucleus can relax to re-establish the Boltzmann distribution. W_0 and W_2 are cases where both the nuclear spins flip simultaneously, and are important only when there is dipole-dipole interaction between A and B, that is, when A contributes to the relaxation of B and *vice versa*.

If the transition ν_A is saturated, the population distributions of the states are altered. The populations of states $\underset{\sim}{1}$ and $\underset{\sim}{2}$ are

Figure 4.7: Solomon diagram to show AB spin system



increased by an amount δ, while those of $\underset{\sim}{3}$ and $\underset{\sim}{4}$ are decreased by the same amount. This does not affect W_1^B, since the difference in population between states $\underset{\sim}{1}$ and $\underset{\sim}{2}$ and between $\underset{\sim}{3}$ and $\underset{\sim}{4}$ is unchanged. It does, however, alter W_0 and W_2. In small organic molecules $W_2 >> W_0$, and so state $\underset{\sim}{1}$ is depopulated with respect to $\underset{\sim}{2}$, and state $\underset{\sim}{4}$ is populated with respect to $\underset{\sim}{3}$. This increases the intensity of signal B by allowing an increased number of transitions $\underset{\sim}{2} \rightarrow \underset{\sim}{1}$ and $\underset{\sim}{4} \rightarrow \underset{\sim}{3}$.

In contrast, in the case of large, slowly tumbling molecules, $W_0 > W_2$, and so irradiation of A leads a depopulation of state $\underset{\sim}{2}$ relative to $\underset{\sim}{1}$ and a population increase in $\underset{\sim}{3}$ relative to $\underset{\sim}{4}$. This decreases the number of transitions $\underset{\sim}{2} \rightarrow \underset{\sim}{1}$ and $\underset{\sim}{4} \rightarrow \underset{\sim}{3}$, giving a smaller signal B (the negative n.O.e.).

This effect is described by Williams and Kalman[192], who show that since W_2 corresponds to a larger energy difference than W_0, molecules with a high frequency of molecular motion will be relaxed more efficiently by W_2 because of the interaction of the molecular motion with the relaxation processes. Conversely, molecules with a low tumbling rate will have more efficient relaxation by W_0. They show that valinomycin, a large glycopeptide antibiotic, gives negative n.O.e.s in $[^2H_6]$-dimethylsulphoxide solution, while Sanders *et al.*[193] found positive n.O.e.s for chlorophylls and metalloporphyrins in $[^2H_6]$-acetone.

The behaviour of alkylcobalamins in ^2H_2O was expected to be intermediate to these two cases, and so negative or positive n.O.e.s might be seen. In molecules of intermediate behaviour, the effects can cancel out, giving vanishingly small n.O.e.s. In fact, small negative effects were observed, showing that alkylcobalamins tumble quite slowly in aqueous solution ($\sim$ 10 mM). When the solvent was changed to $[^2H_4]$-methanol the n.O.e. virtually disappeared but the spectra were better resolved. Cooling the sample to -10 ° slowed the molecular motion down enough to observe a strong negative n.O.e.

4.3.3.b. N.O.e. difference spectra

Originally, n.O.e.s were measured by integration of the signal of interest (B), with and without saturation of the signal corresponding to (A). Changes of less than $\sim$ 5 % could not be detected, and effects due to decoupling and frequency shifts could make interpretation difficult.

The use of FT-NMR spectrometers allowed the development of

n.O.e. difference spectroscopy[194], which overcomes these problems by directly subtracting irradiated and non-irradiated spectra within the spectrometer's computer. The detection limit then depends only on the signal to noise ratio, which can be improved by increasing the number of scans, and on the efficiency of subtraction. The latter is improved by acquiring control and enhanced FIDs alternately (or in alternate blocks of a small number of scans) to minimise the effects of long-term drift of the spectrometer frequency. The difference spectrum is also free from decoupling effects, since the saturation pulse is switched off shortly before data acquisition begins (0.5 ms). Population effects, such as n.O.e., persist until the observe pulse occurs, as they build up and decay relatively slowly, whereas decoupling effects disappear essentially at the instant the decoupler is switched off. Therefore the spectrum contains only differences in line intensity and not in position.

The main feature of an n.O.e. difference spectrum is a large negative signal, corresponding to the pre-irradiated signal (A). The intensity of this signal depends on the extent of saturation of the A multiplet in the pre-irradiated spectrum. The other peaks arise through population changes caused by the saturation of the A protons. These can arise in four ways[190].

Firstly, direct n.O.e. enhancements occur, which are transmitted through space to protons close to protons A, and may be positive or negative, as described above.

Secondly, transmitted enhancements may occur, in which a strongly enhanced proton (B) will transmit a population disturbance to a neighbouring proton (C) by mutual cross-relaxation. In rapidly tumbling molecules this interaction will be small, and will

alternate in sign for each intermediate cross-relaxation, that is, the proton C will show a negative n.O.e., while one affected by C will show a positive one. In practice, it is found that these interactions build up slowly and give very small percentage enhancements.

In contrast, transmitted enhancements in slowly tumbling molecules build up very quickly, and can reach levels comparable to those of direct enhancements. They also have the same sign as the direct effects, both being negative. This spin diffusion can complicate the interpretation but it is minimised by using short pre-irradiation times. Long range effects, possibly due to spin diffusion were seen in some of the cobalamin n.O.e. difference spectra, so only qualitative conclusions could be drawn from the results, rather than precise measurements of interatomic distances.

Thirdly, selective population transfer effects may be seen if the component lines of a pre-irradiated multiplet (A) are partially saturated to different extents. This effect is transmitted through bonds to protons coupled to A, and while it does not alter the total intensity of the signal of a coupled proton, it results in a redistribution of intensity between the lines of the multiplet. This was not seen in the present work, since the irradiated lines were usually singlets, or were fully saturated.

Lastly, if the pre-irradiated proton is exchangeable, saturation may be transferred by chemical exchange. This was not a problem in this work, as no exchangeable protons were irradiated.

4.3.3.c. N.O.e. to assign the methine protons on the corrin system

The unassigned signals between δ 2.6 and 4.5 corresponding to C3-H, C8-H, C13-H and C19-H were assigned, for methylcobalamin, as

follows:

i) First, the signals for C5-CH_3 and C15-CH_3 (δ 2.48 and 2.45, or *vice versa*) were distinguished by irradiation of the methyl groups on the benzimidazole ligand (B5- and B6-CH_3, at δ 2.21). The crystal structure shows that these methyl groups lie closer to C5-CH_3 than to C15-CH_3, and it is likely that this applies also in solution, when the cobalamin is in the base-on form. Irradiation of the signal at δ 2.21 gave a negative n.O.e. (-3.5 %) at δ 2.48, and only -1.4 % at δ 2.45. Therefore C5-CH_3 was assigned to δ 2.48 and C15-CH_3 to δ 2.45. The other enhancements seen on irradiating the B5- and B6-methyl groups were in agreement with those predicted using the assignments of Hill *et al.*[187].

ii) C15-CH_3 (δ 2.45) was next irradiated. The closeness of the C5-CH_3 signal meant that this frequency was also partially saturated. The irradiation produced effects, as expected, on B2-H, C17-CH_3 and C12β-H, and also affected the signals at δ 3.01 (-9.0 %) and δ 4.17 (-1·8 %). C15-CH_3 is nearer to C13-H than any other methine proton, so this is assigned to δ 3.01. The signal at δ 4.17 may be a long range effect on C19-H, or it may be produced by the partial irradiation of C5-CH_3. It could not therefore be assigned from this spectrum.

iii) Irradiation of C12β-CH_3 at δ 0.92 confirmed the assignment of the signal at δ 3.01 as C13-H (-9.4 %). There was also an effect at δ 3.40 (-3.8 %) and this was assigned as C8-H, the effect possibly being transmitted by spin-diffusion *via* C10-H.

iv) Irradiation of C5-CH_3 (δ 2.48) produced an effect at δ 4.17 (-17.9 %) showing that this is C3-H. The expected effects were also seen on C7-CH_3 and B5- and B6-CH_3.

Thus the results of these experiments allowed C3-H (δ 4.17), C8-H (δ 3.40) and C13-H (δ 3.01) to be positively asssigned. The remaining signal in this region, a doublet at δ 3.93 (J = 9.7 Hz) was assigned to C19-H. Decoupling of this signal caused changes at ∿ δ 2.7, indicating the approximate position of C18-H. Further decoupling experiments on the other methine protons showed that C3-H and C13-H are coupled to the region ∿ δ 2.0, and C8-H is coupled to ∿ δ 1.0 and δ 1.9. These give an indication of the chemical shifts of some of the methylene protons on the side chains, but the resolution was not good enough to allow further assignments.

Similar n.O.e. difference experiments were employed to assign the methine protons in (*S*)-dihydroxypropylcobalamin and (*S*)-dihydroxybutylcobalamin. These results are summarised in Tables 4.5 and 4.6, and in more detail in the experimental section. These spectra were obtained in [$^2H_4$]-methanol solution. At 298 K in this solvent, the n.O.e. was not observed, so the samples were cooled to increase the negative n.O.e. by the reduction of the tumbling rate. A series of spectra was obtained for (*S*)-dihydroxypropylcobalamin at various temperatures. Some temperature-dependent shifts were observed, as well as a loss of resolution. In particular, the signals corresponding to C3-H and C19-H changed places on cooling below 283K (Figure 4.8). The temperature-dependent shifts of (*S*)-dihydroxybutylcobalamin were much smaller. When n.O.e. experiments were attempted at 233 K, non-specific effects were seen throughout the spectrum, showing that the tumbling rate had been reduced too far, and spin diffusion effects were becoming dominant. The clearest n.O.e.s were obtained at 263 K (-10 °).

Figure 4.8 Temperature dependence of the chemical shifts of H-3 and H-19 of (S)-dihydroxypropylcobalamin (400 MHz, CD_3OD)

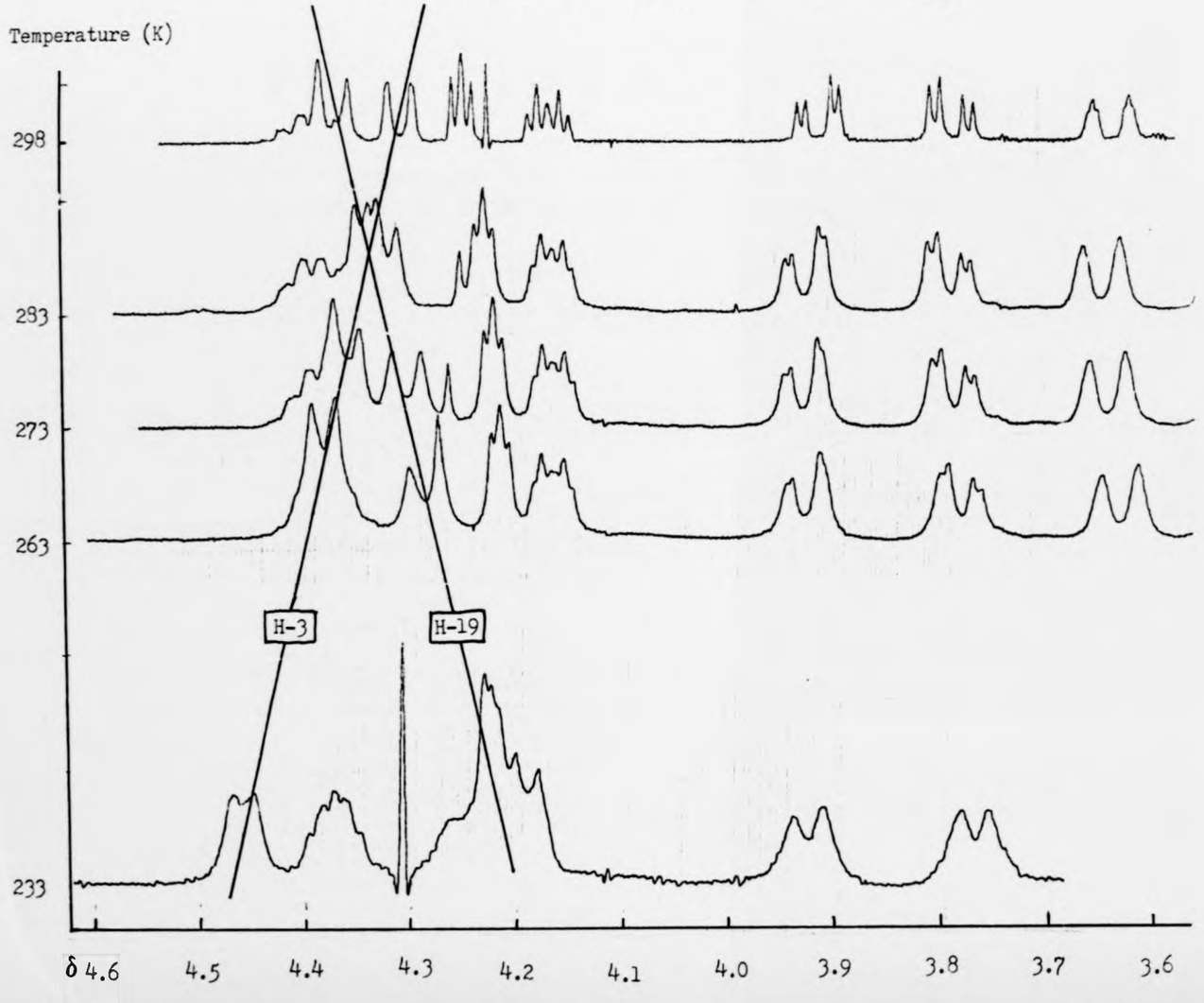


Table 4.4: Methylcobalamin : % n.O.e. results
(Effects > 1.5 % are shown). D_2O, room temperature.

Affected position	δ	Irradiated position (δ) B5,6 (2.21)	C15 (2.45)	C12β (0.92)	C5 (2.38)
B7-H	7.19	-18.0			-1.8
B2-H	6.97		-5.4		-3.6
B4-H	6.28	-21.0	-1.8	-6.3	-5.4
C10-H	5.90			-6.3	
C3-H	4.17		-1.8		-17.9
C19-H	3.95				
C8-H	3.40			-3.8	
C13-H	3.01		-9.0	-9.4	
C18-H	2.6				
C5-CH_3	2.48	-3.5			
C15-CH_3	2.45	-1.4		-2.1	
B6,5-CH_3	2.21				
C7-CH_3	1.76				-6.0
C12α-CH_3	1.39				
C2-CH_3	1.34				-3.0
C17-CH_3	1.34		-4.8		
C12β-CH_3	0.92				
C1-CH_3	0.47				
Co-CH_3	-0.12	-2.1		-2.5	

Table 4.5: (*S*)-Dihydroxypropylcobalamin : % n.O.e. results
(Effects > 1 % are shown) [$^2H_4$]-methanol, 263 K

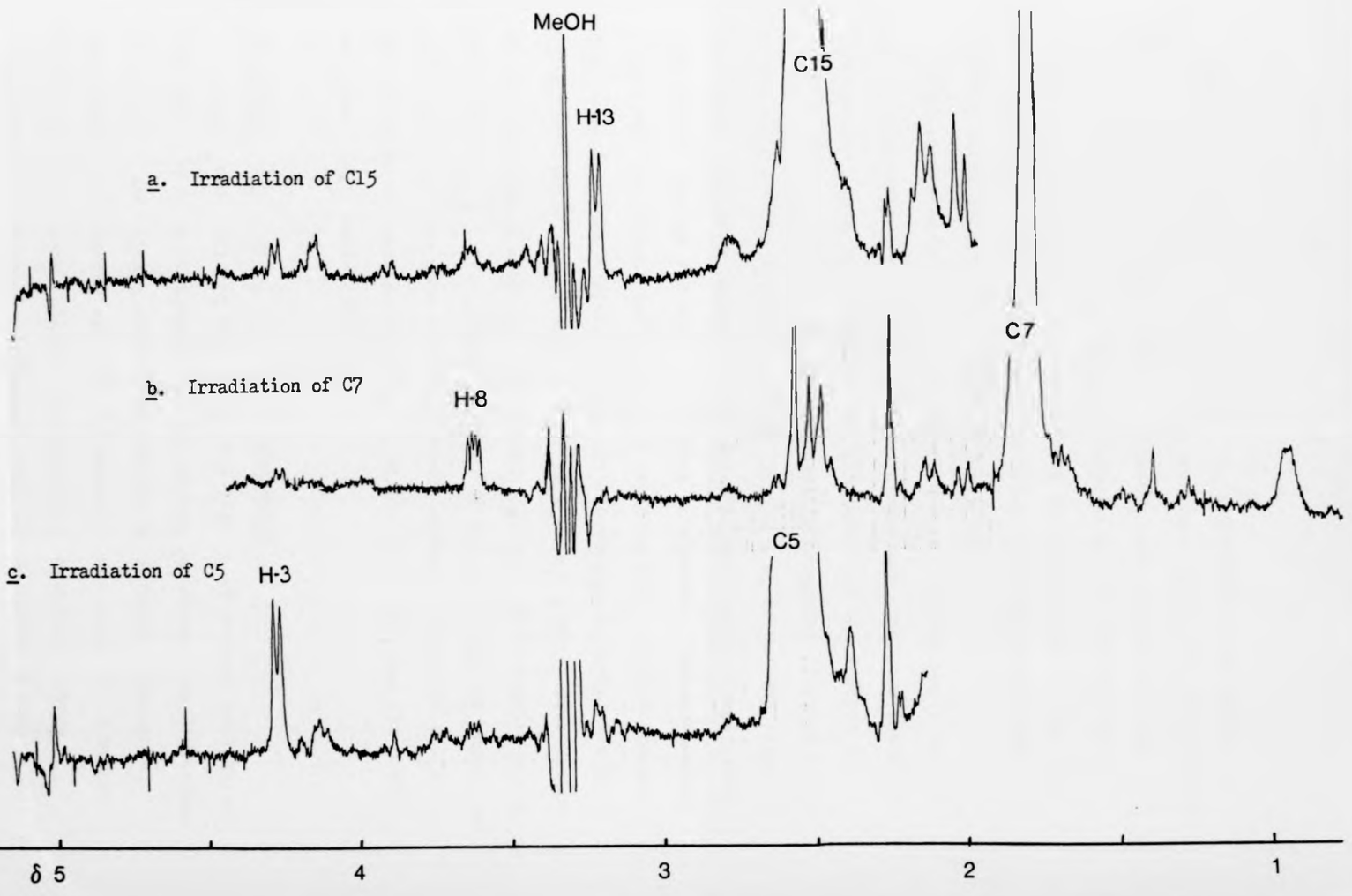
		Irradiated position (δ)				
Affected position	δ	C1 (0.46)	C12β (1.27)	C7 (1.89)	C5 (2.60)	C15 (2.57)
B7-H	7.20					
B2-H	7.18				-2.8	
B4-H	6.48				-3.3	
C10-H	6.14		-5.4	-2.7		
C3-H	4.37	-3.0		-2.7	-15.9	-12.3
C19-H	4.30	-1.5				
C8-H	3.55			-9.2		
C13-H	3.30		-6.9		-11.7	-27.0
C18-H	~2.8	-16.5				
C5-CH_3	2.60			-8.2		
C15-CH_3	2.57		-1.4			
B6-CH_3	2.30					
B5-CH_3	2.29					
C7-CH_3	1.89				-4.1	
C12α-CH_3	1.46		-4.0			
C2-CH_3	1.42	-3.1				
C17-CH_3	1.38					-12.7
C12β-CH_3	1.27					
C1-CH_3	0.48					
*C2'-H	1.67		-4.8			
*C1'-H	0.87				-1.4	

* Possible assignments to protons on alkyl ligand.

Table 4.6: % N.O.e. results for (*S*)-dihydroxybutylcobalamin (Effects > 2 % are shown) [$^2H_4$]-methanol 263 K

		Irradiated position (δ)				
Affected position	δ	C5 (2.56)	C15 (2.53)	C7 (1.86)	C12β (1.12)	C1 (0.52)
B7-H	7.15					
B2-H	7.02	-3.7	-4.3			-4.6
B4-H	6.30	-5.0		-7.1		-6.9
C10-H	6.09			-3.5	-3.2	
C3-H	4.26	-16.3	-4.3			-3.5
C19-H	4.13	-5.0	-4.3			-4.6
C8-H	3.63	-2.5	-2.1	-12.4		
C13-H	3.21		-10.7		-12.9	
C18-H	2.79					-20.8
C5-CH_3	2.56			-7.1		
C15-CH_3	2.54					
B5,B6-CH_3	2.25			-5.9		
C7-CH_3	1.80	-5.0				
C12α-CH_3	1.49				-2.1	
C2—CH_3	1.39					-5.4
C17-CH_3	1.39		-3.6			
C12β-CH_3	1.12					
C1-CH_3	0.53					

N.O.e. difference spectra of (S)-dihydroxybutylcobalamin



For both these compounds, C3-H was identified by the effect on irradiation of C5-CH_3, C8-H by the effect when C7-CH_3 was irradiated, C13-H by the effects when C12 β and C15-CH_3 were irradiated, and C19-H by the effect when C1-CH_3 was irradiated. In the last case, C3-H and C18-H were also affected by the irradiation of C1-CH_3, but C3-H had already been identified, and C18-H had a quite different chemical shift (δ 2.6) from C19-H (δ 4.2-4.4).

The assignments of the methine protons in [$^2H_4$]-methanol were therefore unambiguous at -10 °, and by comparison, the room temperature spectra could be assigned. The assignment of the spectra in D_2O was made by a comparison of the coupling constants (J C19-H ∿ 11 Hz, JC3-H ∿ 9 Hz, JC8-H ∿ 5, 11 Hz, JC13-H ∿ 2, 10 Hz) and relative chemical shifts with the methanolic spectra, and similar comparisons were used to assign the spectra of other cobalamins. The assignments of the methine protons are summarised in Table 4.3 . An attempt was made to confirm the assignments for (*R*)-dihydroxypropylcobalamin by n.O.e. difference spectroscopy at 5 ° in D_2O at 300 MHz. Irradiation of the signal at δ 2.55 (C15- and C5-CH_3) gave a small negative n.O.e. on the signal at ∿ δ 4.1, that is C3-H, as expected, but the resolution of the spectrum was poor and the expected effect on C13-H (∿ δ 3.3) was not seen. Irradiation of C1-CH_3, C12β-CH_3, C7-CH_3 and C10-H did not give recognisable effects.

4.3.4 Assignment of the alkyl ligands in cobalamins

The signals arising from the alkyl ligands of several cobalamins were assigned by a combination of decoupling and decoupling difference techniques. Decoupling difference spectra were found to be useful when coupled resonances were buried beneath other signals. A Bruker microprogram was used, which accumulated 8-12 transients while irradiating at the chosen frequency, then accumulated the same number

of transients with irradiation at an off-resonance control frequency, subtracting the resulting FIDs. The cycle was repeated until the required number of scans had accumulated.

Examples of spectra obtained by these techniques are shown in Figure 4.9, and assignments of the chemical shifts and coupling constants of various alkyl ligands are listed in Table 4.7.

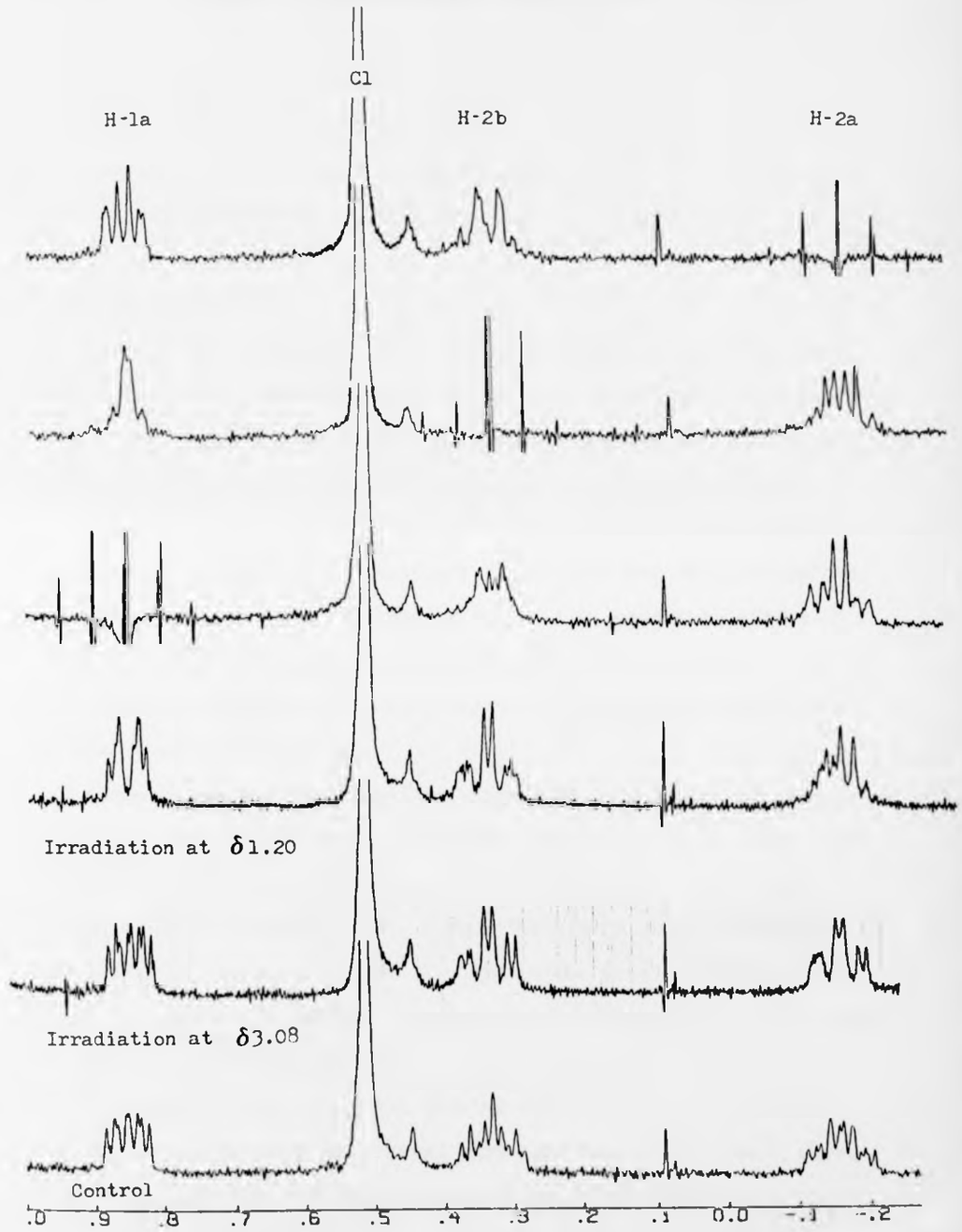
4.3.5 Discussion of the structural and conformational information obtained from ^{1}H NMR spectra

^{1}H NMR spectra give information about the structure and comformation of cobalamins in solution, and have given insights into how the structure compares with that in the crystal. Chemical shifts and coupling constants are, however, dependent on a wide range of steric and electronic factors, so a firm interpretation of these parameters in terms of one fixed structure cannot be made. Nevertheless, comparison of the spectra of a range of cobalamins (Tables 4.2, 4.3, 4.7) does show that there are certain trends, which are discussed here, that give evidence of certain conformations being preferred over others. NMR spectra give, of course, a time-averaged picture of the molecule, so the data must be interpreted as showing an equilibrium between all the possible structures which the cobalamin adopts in solution.

4.3.5.a) The ribose, propanolamine and 5,6-dimethylbenzimidazole groups

Inspection of the chemical shifts and coupling constants of the signals associated with this part of the molecule in various alkylcobalamins, showed that, insofar as these parameters reflect the conformation of the cobalamin in solution, only small changes occur upon alteration of the alkyl ligand (Table 4.3.a.). This is in agreement with the data of Hill *et al.*[187], who found that the

Figure 4.9 Double irradiation to assign the resonances of the alkyl ligand of (S)-dihydroxybutylcobalamin



coupling constants of these moieties in methyl- and adenosylcobalamin were very similar, but showed slight changes in cyano- and aquo-cobalamin, consistent with a shortening of the Co-N (benzimidazole) bond in the latter two compounds. The coupling constants were also shown to be consistent with the conformation in the crystal state, indicating that there are no large conformational differences between the solution and crystal structures.

b) The corrin system

The chemical shifts of the methyl and methine protons on the corrin ring were seen to be more variable, in general, than those associated with the lower (α) axial ligand. This is to be expected, as changes in the alkyl ligand are likely to have a greater effect on the closer parts of the molecule. There is again, in general, an agreement with the crystal structure, where this has been determined, which also supports the findings of ref.87., in which the shifts induced by Pr^{3+} binding to the phospho-diester group of several cobalamins are approximately proportional to the distances measured in the crystal structures.

Some of the observed chemical shifts (Table 4.2) can be explained in terms of the presumed structures of the cobalamins. For instance, as already mentioned, C1-CH_3 is at high field (δ 0.4 - 0.6) while C7-CH_3 is at low field (δ 1.8 - 1.9). These both project towards the α-face of the corrin, and are in the shielded and deshielded regions of the 5,6-dimethylbenzimidazole base, respectively. The other methyl groups on this face, C2 and C12α, have chemical shifts of δ ∿ 1.4 and ∿ 1.5 respectively, showing that they are not so much affected by the base. The crystal structure of the coenzyme[33] shows that C2 and C12α are both pseudo-equatorial, which moves them further

away from the region influenced by the base. C1, in contrast, is almost axial, and is positioned near to the aromatic system.

The corresponding group on ring B is the propionamide-side chain on C8. In methyl cobalamin, decoupling of C8-H (δ 3.40) causes an effect at δ 1.0, suggesting that at least one of the protons on this propionamide resonates at such high field, which is among the highest shown by any of the methylene signals in the molecule.

The methyl and methine groups on the β-face of the corrin show some quite marked variations, depending on the alkyl ligand and the solvent.

The methine protons C3-H, C8-H, C13-H and C19-H (Table 4.3b) seem to be the most sensitive to changes in these parameters, and therefore give the most useful information. They are shown in Figure 4.11.

C3-H, on ring B, shows the least variation of the methine protons. For cobalamins in aqueous solution it gives signals between δ 4.03 and 4.08, except for methylcobalamin (δ 4.17). Inspection of models, and of the three crystal structures of alkylcobalamins, shows ring B to be in the most crowded part of the molecule, that is, the region in which alkyl ligand will be most sterically compressed, therefore the alkyl ligands tend to point towards rings C and D, and do not affect C3-H much. In methanolic solution the signal for C3-H moves downfield, to δ 4.2 - 4.3.

C8-H shows similar variations with solvent, moving from δ 3.40 - 3.45 in aqueous solution (with the exceptions discussed below) to δ 3.55 - 3.67 in methanolic solution. The only alkylcobalamins not conforming to these values are (*S*)-hydroxypropyl- and (*S*)-dihydroxypropylcobalamin and one isomer (probably (*S*)-) of 2-hydroxy-2-cyclohexylethyl- and 2-hydroxy-2-t-butylethylcobalamin, where C8-H

gives a signal at ∿ δ 3.20 in aqueous solution. These compounds are more fully discussed below, where C19-H is considered.

C13-H and C19-H are little affected by changes in the solvent, except in the case of (*S*)-dihydroxypropylcobalamin, where both signals are shifted downfield (by ∿ 0.1 and ∿ 0.2 p.p.m. respectively) on changing from water to methanol. The effect of methanol on the spectra of other β-hydroxypropylcobalamins was not studied. C13-H varies between δ 3.12 and 3.30 except in the cases of methylcobalamin (δ 3.01) and adenosylcobalamin (δ 2.90). The signal from adenosylcobalamin can be explained by reference to the crystal structure[33], which shows that the adenine base of the β-ligand lies above ring C. Substituents on this ring should therefore be shielded, and C13-H and C12β-CH_3 are indeed shifted upfield by ∿ 0.3 p.p.m., compared to other alkylcobalamins. This similarity with the conformation found in the crystal, is supported by the work of Hogenkamp *et al.*[195] who measured the relaxation times of the carbon bonded to cobalt in various ^{13}C-enriched cobalamins. In the case of adenosylcobalamin, T_1 is very short, in methylcobalamin it is longer, and ethylcobalamin gives an intermediate value. T_1 gives a measure of the mobility of a particular atom, so these results suggest that rotation about the Co-C bond becomes progressively more difficult as the alkyl ligand becomes larger.

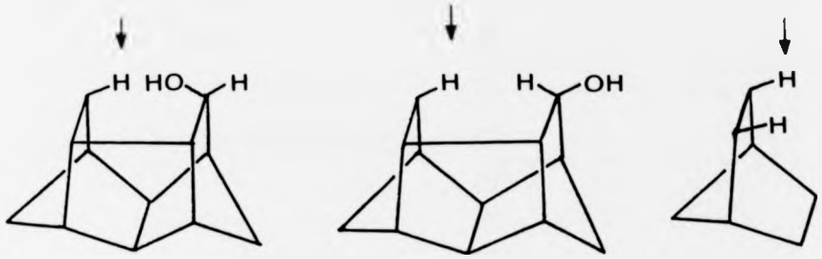
Of the four methine protons on the β-face, C19-H shows the widest variation of chemical shifts (δ 3.94 - 4.60). These values fall into three distinct groups, δ 3.94 (methylcobalamin), δ 4.10 - 4.37, and δ 4.57 - 4.60. The abnormally low field signals belong to (*R*)-2,3-dihydroxypropyl- and (*R*)-2-hydroxypropylcobalamin, and the major isomer in each case of 2-hydroxy-2-t-butylethylcobalamin and 2-hydroxy-2-cyclohexylethylcobalamin, produced by the reaction

of cobalamin(I) with t-butyloxirane and cyclohexyloxirane, respectively (section 3.3.2). The *R*-isomer is the major isomer from the reactions of glycidol and methyloxirane with cobalamin(I), so it is reasonable to suppose that the *R*-isomer is the major isomer from the reaction of other monosubstituted oxiranes. Thus it appears that C19-H is unusually deshielded in the case of (*R*)-2-hydroxyalkylcobalamins. An explanation is suggested by comparison of the crystal structures of (*R*)- and (*S*)-2,2-dihydroxypropylcobalamin (Chapter 5). It can be seen that the *S*-isomer makes a hydrogen bond between C2-OH and the acetamido- side chain of ring B. Even if the hydrogen bond does not persist in solution, three pieces of evidence suggest that the conformation is not very different. Firstly, a small n.O.e. was seen between C2-H of the alkyl ligand and C12β-CH_3 of the corrin. Secondly, the coupling constants on the alkyl ligand suggest a similar conformation (discussed below), and thirdly, C8-H shows upfield shifts ($\sim$ 0.2 p.p.m.) in all (*S*)-2-hydroxyalkylcobalamins compared to other alkylcobalamins, which suggests that there is still some interaction between the alkyl ligand and ring B. The -CH_2OH group therefore lies in the channel between C and D rings (Figure 5.4).

The *R*-isomer, however, cannot make such a hydrogen bond without unfavourable steric interactions occurring between the -CH_2OH group and the corrin in the region of ring C. In this isomer, therefore, the crystal structure shows the -CH_2OH group pointing up towards ring C. Inspection of models shows that this minimises steric interactions between the alkyl ligand and the corrin, but it also brings C2-OH into close contact with C19-H (Figure 5.2). This causes Van der Waal's deshielding[138] of C19-H, in which groups which are held close together deshield each other by mutual distortion of their electron clouds. An example is shown in figure 4.14.[196].

Figure 4.14

$\Delta\sigma$ = -2.4 p.p.m. $\Delta\sigma$ = -1 p.p.m. $\Delta\sigma$ = 0



σ is the shielding constant.

A negative value for $\Delta\sigma$ signifies a shift to lower field.

The consistent shift of C19-H to low field in the case of (*R*)-2-hydroxyalkylcobalamins suggests that all these compounds have similar conformations.

Little information can be gained about the conformations of other alkylcobalamins from the corrin resonances as they show no readily discernible patterns of shifts.

c) The alkyl ligands

Analysis of the chemical shifts and coupling constants of the alkyl ligands (Table 4.7) gives information about the conformation of the ligands in solution.

Table 4.7: Alkyl ligands

Solvent	Alkyl group	δ (p.p.m.)	J (Hz)
D_2O	Methyl	-0.12	
D_2O	Ethyl	C1α = 1.4 C1β = 0.64 C2 = 0.57	JC1α-C1β = 7.6 Hz C1α-C2 = 7.6 C1β-C2 = 7.6
D_2O	(*R*)-Hydroxypropyl	C1α = 0.48 1β = 1.38 C2 = 1.93 C3 = 0.58	C1α-C1β = 8.5 C1α-C2 = 2.5 C1β-C2 = 6.9 C2 -C3 = 6.3
D_2O	(*S*)-Hydroxypropyl	C1α ∿ 0.5 1β = 1.3-1.6 C2 = 1.62 C3 = 0.50	C1α-C1β ∿ 7 C1α-C2 ∿ 2 C1β-C2 ∿ 7 C2 -C3 = 5.8
D_2O	(*R*)-Dihydroxypropyl	C1α = 0.48 C1β = 1.13 C2 = 1.66 C3α = 2.76 C3β = 2.86	C1α-C1β = 9.1 C1α-C2 ∿ 2 C1β-C2 = 7.3 C2-C3α = 7.3 C2 -C3β = 4.2 C3α-C3β = 11.2
D_2O	(*S*)-Dihydroxypropyl	C1α = 0.84 C1β = 1.54 C2 = 1.62 C3α = 2.72 C3β = 2.81	C1α-C1β = 8.8 C1α-C2 = 3.7 C1β-C2 = 5.6 C2 -C3α = 4.0 C2 -C3β = 6.9 C3α-C3β = 11.2
CD_3OD	(*S*)-Dihydroxybutyl	C1α = 0.86 C1β = 1.23 C2α = -0.15 C2β = 0.34 C3 = 3.08 C4 = ∿3.15	C1α-C1β = 6.9 C1α-C2α = 5.0 C1α-C2β = 12.5 C1β-C2α = 13 C1β-C2β = 5.0 C2α-C2β = 13 C2α-C3 = 4.7 C2β-C3 = 7.4 C3 -C4α ∿ 5 C3 -C4β ∿ 5 C4α-C4β ∿ 12.5
CD_3OD	(*R*)-Dihydroxybutyl	C1α = 0.47 C1β ∿ 1.58 C2α = -0.15 C2β = 0.34 C3 = ∿3.05 C4α = ∿3.2 C4β = ∿3.2	C1α-C1β ∿ 12 C1α-C2

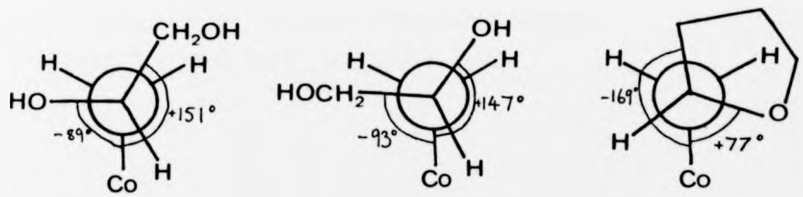
Table 4.7: contd.

Solvent	Alkyl group	δ (p.p.m.)	J (Hz)
CD_3OD	(*S*)-Dihydroxypentyl	C1α = 0.60 C1β = 1.28 C2α = -0.25 C2β = 0.46 C3α,β = 1.02 C4 = 3.25 C5α,β = 3.18	
D_2O		C1α = 0.54 C1β = 0.85? C2α = -0.37 C2β = 0.40 C3α,β = 1.02? C4 = 3.32 C5α = 3.16 C5β = 3.27	
D_2O	(*R*)-Dihydroxypentyl	C1α = 0.54 C1β = ? C2α = -0.24 C2β = 0.30 C3α,β ∿ 1.02 C4 ∿ 3.32 C5α,β ~ 3.15-3.3	
CD_3OD	(*R* + *S*)-Dihydroxyhexyl probable assignments	C1α = 0.52 C1β = 0.19 C2α = -0.44 C2β = 0.19 C3-C4 = ∿0.6-1.5 C5-C6 = 3.3-3.55	
	n-Hexyl probable assignments	C1 1.42, -0.13 C2 -0.81, 0.31 C3 C4 0.6, 1.4? C5 C6 = 0.64 (t, 3H)	C5-C6 = 7
	n-Octyl	C1,C2 ∿ 0.19, -0.4 C8 0.77 (t, 3H)	C7-C8 = 6.5
D_2O	2-7-Butyl-2-hydroxyethyl major isomer (*R ?*)	C1α = 0.71 C1β = 0.96 C2 = 1.3-1.4 C4 = 0.43	C1α-C1β = 9.5 C1α-C2 ∿ 0-2 C1β-C2 ∿ 7
0.1 M DCl	Neopentyl	C1α = 0.92 C1β = ? C3 = -0.11	

i) 2-Hydroxyalkylcobalamins

These are considered in most detail, because of the availability of the crystal data for (*R*)- and (*S*)-2,3-dihydroxypropylcobalamin and because the relative simplicity of their NMR spectra allowed the coupling constants to be determined with greater accuracy. Figure 4.15 shows the torsion angles found in the crystal structures for the hydrogens on C1 and C2 of these two cobalamins and the equivalent hydrogens in adenosylcobalamin, which has the *S*-configuration at the β-carbon.

Figure 4.15 Newman projections along C2-C1 of (*R*)- and (*S*)-2,3-dihydroxypropylcobalamin and adenosylcobalamin.



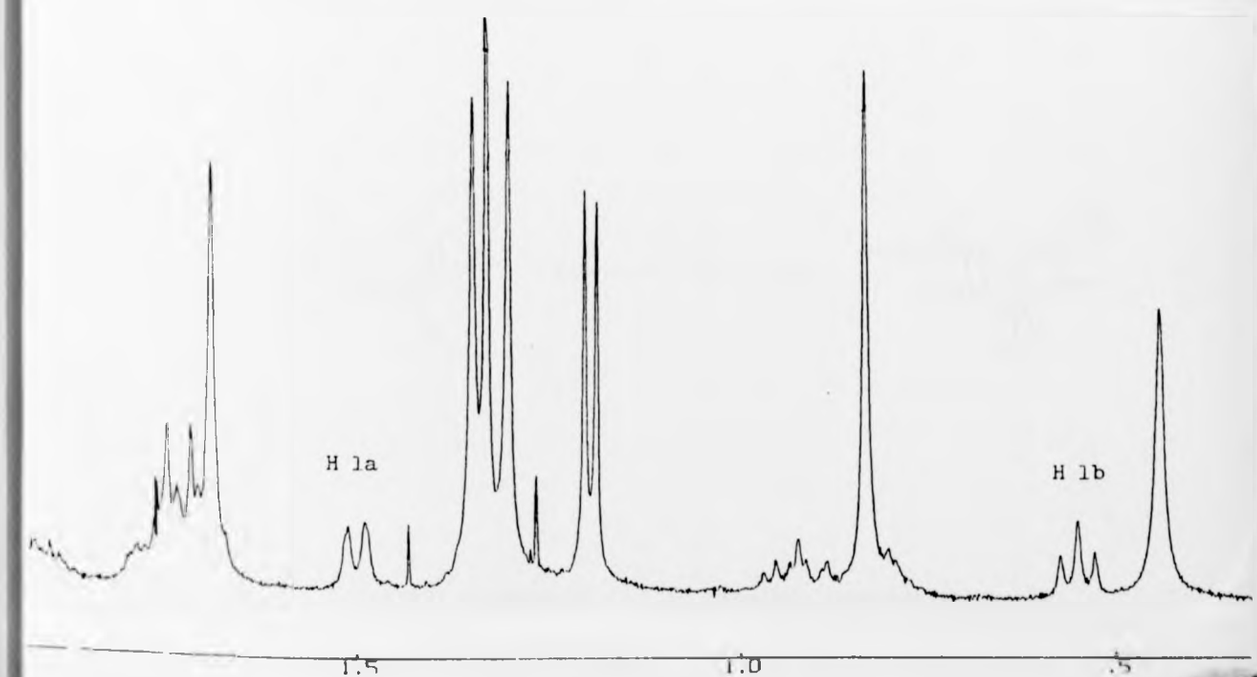
R *S* Adenosyl *(S)*

Table 4.8 shows the torsion angles and expected and actual coupling constants. The expected J values were calculated on the basis of the Karplus-Conroy curve[138]. It can be seen that while the coupling constants of (*R*)-dihydroxypropyl- and adenosylcobalamin fit reasonably well with the calculated values, those for the (*S*)-isomer

Table 4.8: Calculated and observed coupling constants for three alkylcobalamins

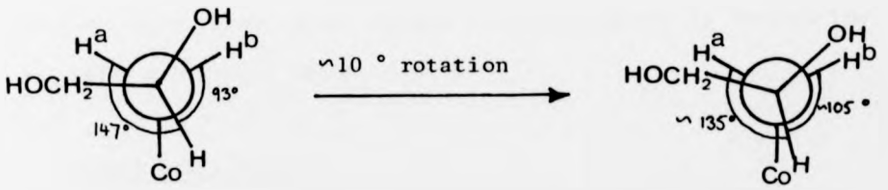
Alkyl group	Torsion angle	$J_{expected}$ Hz	$J_{observed}$ Hz	Assignment δ p.p.m.
(*R*)-Dihydroxypropyl				
H-1a - H 2	150.9 °	8 - 11	7.3	1.13
H-1b - H 2	89.1 °	0 - 3	∿ 2	0.48
(*S*)-Dihydroxypropyl				
H 1a - H 2	146.7 °	8 - 11	5.6	1.54
H 1b - H 2	93.3 °	0 - 3	3.7	0.84
Adenosyl				
H 1a - H 2	77 °	0 - 4	∿ 2	1.50
H 1b - H 2	169 °	10 - 14	8.8	0.56

High-field region of adenosylcobalamin spectrum showing H 1a and H 1b (400 MHz, D_2O)



do not show such agreement. Rotation about C1-C2 by ∿ 10 ° gives torsion angles H1a-H2 ≈ 135 °, H1b-H2 ≈ 105 ° which give expected coupling constants in agreement with those observed. This rotation may be expected to occur in (*S*)-dihydroxypropylcobalamin, as the alkyl ligand is held in a particular arrangement in the crystal, by a hydrogen bond from C2-OH of the alkyl-group, to the acetamido-side chain on ring B. Disruption of this hydrogen bond when the cobalamin is in solution, could lead to a change in conformation of the alkyl ligand, to relieve steric interactions which in the crystal are outweighed by the presence of the hydrogen bond. The steric interactions between the $-CH_2OH$ group and the corrin ring are relieved by a small rotation about C1-C2, although this increases the eclipsing interactions between the groups on C1 and C2 (Figure 4.16). The observed conformation is presumably one which gives the best compromise between these interactions.

Figure 4.16. Structure of (*S*)-dihydroxypropylcobalamin



crystal structure possible solution structure

This analysis allows the diastereotopic protons on C1 to be assigned for (*R*)- and (*S*)-dihydroxypropylcobalamin. In both cases, the one with the larger coupling constant is at lower field, so can be assigned to be H1a in Figure 4.15, or Pro-*S*, while the other proton, at higher field, is pro-*R*. This is in qualitative agreement with the assignment for adenosyl cobalamin, where synthesis from (5'-*R*)-[5'-^{2}H]adenosine allowed the assignment of the pro-*R* proton at δ 0.58 and the pro-*S* proton at δ 1.56.

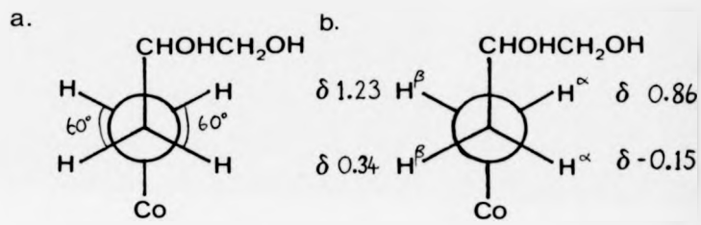
Other β-hydroxyalkylcobalamins were in qualitative agreement with these findings. (*R*)-2-Hydroxypropylcobalamin has coupling constants C1a-C2 = 6.7 Hz and C1b-C2 = 2.5 Hz, and (*R*)-2-hydroxy-2-t-butylethylcobalamin has C1a-C2 ≃ 7 Hz, and C1b-C2 < 2 Hz, both similar to those in (*R*)-dihydroxypropylcobalamin. (*S*)-2-Hydroxypropylcobalamin gives rather indistinct coupling constants, but the signal at δ 1.54 (H1a) has $J_{1a-2} \simeq 7$ Hz, and that at δ ∿ 0.5 probably has $J_{1b-2} \simeq$ 2-3 Hz. In all cases the signal at higher field has the smaller coupling constant, so was assigned to the pro-*R* proton. This shielding of the pro-*R* over the pro-*S* proton, could be because when the alkyl ligand is towards the less hindered C-D side of the molecule, the pro-*R* proton is more nearly over the conjugated part of the corrin chromophore, whereas the pro-*S* proton is towards the saturated A-D link (Figures 5.2, 5.4).

ii) Other alkyl ligands

Table 4.7 shows the chemical shifts and coupling constants for other alkyl ligands. In cases where the alkyl chain is more than four carbons in length, the splitting patterns are complicated, and while decoupling can show the positions of coupled protons, the coupling constants could not be determined. The only alkyl chain

fully assigned was in (*S*)-4,5-dihydroxybutylcobalamin. This has coupling constants J(1α - 2α) = 4.7, J(1α - 2β) = 13.1, J(1β - 2α) = 12.6 and J(1β - 2β) = 4.7 Hz. This is consistent with a staggered conformation (Figure 4.17a) with the alkyl chain pointing up to minimise interactions with the corrin.

Figure 4.17. Conformation of (*S*)-dihydroxybutylcobalamin



The only assignment of chemical shifts that can be made with certainty is that H1α is closer to H2α than it is to H2β. If, however, the higher field signal is the pro-*R* proton, by analogy with 2-hydroxyalkylcobalamins, then the assignments in Figure 4.17b can be made. The alternative possibility is obtained by exchanging the α and β assignments on each carbon.

It can be seen that the protons on carbon-2 are more shielded than those on carbon-1. This is a general feature of alkylcobalamin spectra, and except in the cases of methylcobalamin and neopentylcobalamin, whenever a signal upfield of TSS was assigned, it was found to be from a proton on carbon-2 of the alkyl ligand (that is,

in dihydroxybutyl, dihydroxypentyl-, and hexylcobalamin, and, probably, in n-octyl- and dihydroxyhexylcobalamin as well). Inspection of models shows that these protons are much nearer to the corrin system than are any others and so are likely to be shielded to a greater extent.

4.4 Experimental Section

4.4.1 UV-visible spectra

These were obtained in aqueous solution, using doubly-distilled water. The alkylcobalamin (2 - 3 mg) was accurately weighed, and dissolved in water (100 ml) in a volumetric flask. The spectra were obtained in 1 cm quartz cells, on a Shimadzu UV 365 UV-visible spectrometer.

Exposure of the solution to light for a few minutes gave hydroxocobalamin, and the subsequent addition of a crystal of potassium cyanide gave, in a few hours, dicyanocobalamin. This has an extinction coefficient of 30.8 x 10^3 M^{-1} cm^{-1}, at 368 nm, which allows the concentration of the cobalamin solution to be calculated.

4.4.2 IR spectra

The IR spectra were obtained in aqueous solution using CaF_2 cells, or as nujol mulls between NaCl plates. The spectra were recorded on an Nicolet FT-IR spectrometer, or on a Perkin-Elmer 257.

4.4.3 ^1H FT-NMR spectra

The alkylcobalamin ($\sim$ 7 mg, 5 x 10^{-3} mmol) was dissolved in D_2O, or [$^2H_4$]-methanol (0.5 ml) to give a $\sim$ 10 mM solution.

The ^1H NMR spectra were obtained on a Bruker WH 400 spectrometer, operating under ASPECT 2000 control, using a spectral width of 5376 kHz with 32 K data points. This gave an acquisition time of 3.05 s, and a digital resolution of 0.328 Hz per point.

^1H NMR spectra in D_2O were run with an automatic solvent suppression program (Bruker microprogram MULPKSUP), which reduced the intensity of the HOD peak, allowing the FID to be accumulated at higher

receiver gain, without overloading the memory. The program consisted of irradiation of the solvent peak, (18 L, 2.5 s), a delay (0.5 ms) with the decoupler gated off, then the FID was accumulated before the solvent peak had recovered. The cycle was repeated 64 - 400 times.

The resulting FID was usually subjected to Gaussian multiplication (GM = 0.2 - 0.5, LB = -1 - -2) in order to improve the resolution, although this was at the expense of the signal to noise ratio. Therefore in spectra with low signal to noise, an exponential multiplication was applied (LB $\sim$ 0.2 - 0.8 Hz).

Decoupling experiments were done either directly, with continuous-wave irradiation of the chosen frequency, or in the difference mode, using a Bruker microprogram (DECDIFF). In this, 8-12 transients were recorded with decoupling of the chosen frequency, then the same number with irradiation of an off-resonance frequency, set as close as possible to the on-resonance frequency. The two sets of spectra were subtracted, and the cycle was repeated until the required number of scans had accumulated. The decoupler power was set at 18-23 L, low enough to be selective, while saturating the required signal.

N.O.e. experiments were run using an automatic Bruker microprogram (NOEDIFF). This repeats the basic sequence of pre-irradiation of the chosen signal at frequency 02, with decoupler power 23 L, for 2.5 s, then a delay (0.5 ms), observation pulse, and acquisition of the FID. When 8-12 transients had been collected and summed, 02 was changed to an off-resonance frequency, and an equal number of transients were collected and subtracted from the accumulated data. 02 was then returned to the original frequency and the cycle was repeated, typically 100-200 times.

After the experiment, the file was transformed after exponential multiplication (LB = 0.3 Hz).

4.4.4a. Methylcobalamin

Decoupling experiments

	Irradiation (δ)	effect (δ)	assignment
i)	R1-H (5.90, d)	4.23 dd → d	R2-H
ii)	R2-H (4.71, ddd)	4.23 dd → d 4.08 ddd → dd	R2-H R4-H
iii)	R4-H (4.08, dd)	3.90 dd → d 3.74 dd → d 4.71 ddd → dd	R5α-H R5β-H R3-H
iv)	R5α-H (3.90, dd) and C19-H (3.94, d)[a]	3.90 dd → d 4.08 ddd → dd ∿ 2.6	R5β-H R4-H C18-H
v)	Pr3-CH_3 (1.22, d)	4.33 dddq → ddd	Pr2-H
vi)	Pr2-H (4.33, dddq)	3.56 dd → d 3.11 dd → d 1.22 d → s	Pr1α-H Pr1β-H Pr3-CH_3
vii)	Pr1β (3.11, dd)	3.56 dd → d 4.33 dddq → ddq	Pr1α-H Pr2-H
viii)	C3-H (4.17 dd)[a]	∿ 2.0	propionamide on ring A
ix)	C8-H (3.40, dd)[a]	∿ 1.9 ∿ 1.0	propionamide on ring B
x)	C13-H (3.01 d(d))[a]	∿ 2.0	propionamide on ring C
xi)	(1.0, m)	3.40 dd → d	C8-H
xii)	(2.0, m)	4.17 dd → 3.01 dd → (d)	C3-H C13-H
xiii)	C18-H (2.6, m)[a]	3.94 d → s ∿ 2.0	C19-H ring D propionamide

[a] C3-H, C8-H, C13-H, C19-H were assigned by the n.O.e. experiments.

4.4.4b N.O.e. difference experiments

Methylcobalamin, D_2O, room temperature

Irradiation δ	Effects δ (relative enhancement, assignment)
2.21 (B5- and B6-CH_3)	-0.21 (-2.1 %, Co-CH_3), 0.47 (-1.2 %, C1-CH_3), 2.45 (-1.4 %, C15-CH_3), 2.48 (-3.5 %, C5-CH_3), 6.28 (-21 %, B4-H), 7.19 (18 %, B7-H).
2.45 (C15-CH_3)	-0.21 (-1.2 %, Co-CH_3), 0.92 (-1 %, C12β-CH_3), 1.34 (-4.8 %, C17 or C12α-CH_3), 2.21 (-2.3 %, B5, B6-CH_3), 3.01 (-9.0 %, C13-H), 4.08 (-3.6 %, R4-H), 4.17 (-1.8 %, C3-H), 6.28 (-1.8 %, B4-H), 6.97 (-5.4 %, B2-H).
0.92 (C12β-CH_3)	-0.12 (-2.5 %, Co-CH_3), 0.47 (-1 %, C1-CH_3), 2.21 (-1.4 %, B5,6-CH_3), 2.45 (-2.1 %, C15-CH_3), 2.48 (-1.0 %, C5-CH_3), 3.01 (-9.4 %, C13-H), 3.40 (-3.8 %, C8-H), 5.90 (-6.3 %, C10-H), 6.28 (-6.3 %, B4-H).
2.38 (C5-CH_3)	-0.12 (-1.2 %, Co-CH_3), 1.34 (-3.0 %, C2-CH_3), 1.34 (-3.0 %, C2-CH_3), 1.74 (-6.0 %, C7-CH_3), 4.17 (-17.9 %, C3-H), 6.28 (-5.4 %, B4-H), 6.97 (-3.6 %, B2-H), 7.19 (-1.8 %, B7-CH_3).

4.4.5 (*S*)-2,3-Dihydroxypropylcobalamin, [$^2H_4$]-methanol, 263 K

Irradiation δ	Effects δ (relative enhancement, assignment)
0.46 (C1-CH_3)	1.39 (-3.1 %, C2-CH_3), ∿ 2.8 (-18.6 %, C18-H), 4.28 (-1.5 %, C19-H), 4.38 (-3.0 %, C3-H).
1.31 (C12β-CH_3)	1.46 (-4.0 %, C12α-CH_3), 1.68 (-4.8 %, C2-H), 2.57 (-1.4 %, C15-CH_3), 3.26 (-6.9 %, C13-H), 6.08 (-5.4 %, C10-H).

Irradiation δ	Effects δ (relative enhancement, assignment)
1.67 (C2-H on alkyl ligand)	No identifiable n.O.e.
1.88 (C7-CH_3)	2.60 (-8.2 %, C5-CH_3), 3.53 (-9.2 %, C8-H), 4.38 (-2.7 %, C3-H), 6.07 (-2.7 %, C10-H).
2.57 (C15-CH_3)	1.45 (-12.7 %, C17-CH_3), 3.27 (-27.0 %, C13-H), 4.37 (-12.3 %, C3-H) also some other effects due to partial irradiation of C5-CH_3.
2.60 (C5-CH_3)	0.87 (-1.4 %, C1-H?), 1.88 (-4.1 %, C7-CH_3), 3.27 (-11.7 %, C13-H), 4.38 (-15.9 %, C3-H), 6.36 (-3.3 %, B4-H), 7.07 (-2.8 %, B2-H), also some other effects due to partial irradiation of C15-CH_3.
6.07 (C10-H)	1.46 (-2 %, C12α-CH_3), 3.53 (-11 %), C8-H).

4.4.6 (*S*)-3,4-Dihydroxybutylcobalamin [$^2H_4$]-methanol 263 K

Irradiation δ	Effects δ (relative enhancement, assignment)
2.55 (C5-CH_3)	1.81 (-5.0 %, C7-CH_3), 3.62 (-2.5 %, C8-H), 4.12 (-5.0 %, C19-H), 4.27 (-16.3 %, C3-H), 6.30 (-5.0 %, B4-H), 7.02 (-3.7 %, B2-H).
2.53 (C15-CH_3)	1.39 (-3.6 %, C17-CH_3), 2.36 (-6.4 %, 3.20 (-10.7 %, C13-H), 4.13 (-4.3 %, C19-H), 4.26 (-4.3 %, C3-H), 7.02 (-4.3 %, B2-H).
1.86 (C7-CH_3)	0.95 (-28.9 %,), 2.25 (-5.9 %, B5,6-CH_3), 2.56 (-7.1 %, C5-CH_3), 3.62 (-12.4 %, C8-H), 6.06 (-3.5 %, C10-H), 6.30 (-7.1 %, B4-H).
1.12 (C12β-CH_3)	1.49 (-2.1 %, C12α-CH_3), 3.30 (-12.9 %, C13-H), 6.06 (-3.2 %, C10-H).

Irradiation δ	Effects δ (relative enhancement, assignment)
0.52 ($C1-CH_3$)	1.39 (-5.4 %, $C2-CH_3$), 2.71 (-20.8 %, C18-H), 4.10 (-4.6 %, C19-H), 4.26 (-3.5 %, C3-H), 6.30 (-6.9 %, B4-H), 7.02 (-4.6 %, B7-H).

CHAPTER 5

X-RAY CRYSTALLOGRAPHY OF COBALAMINS AND RELATED COMPOUNDS

CHAPTER 5

X-RAY CRYSTALLOGRAPHY OF COBALAMINS AND RELATED COMPOUNDS

5.1 Introduction

X-ray crystallography has played an important part in the study of Vitamin B_{12}. It was used to elucidate the structure of cyanocobalamin[29,30] and adenosylcobalamin (the coenzyme)[32,33]. It has also been used extensively since then to study a wide range of corrinoids and their analogues, and to explore the geometry around the central cobalt atom. Since the homolysis of the cobalt-carbon bond seems to be an essential step in the enzymic reactions, it is obviously important to study this region in detail. It must be remembered, however, that the conformation in the crystal may be different from that in solution, or when the coenzyme is bound to an enzyme. Very little is known about the binding of adenosylcobalamin to enzymes. In particular, it is not known whether the 5,6-dimethylbenzimidazole base is coordinated or not, although modifications in the base lead to a loss of activity.

Surprisingly, adenosylcobalamin was the only cobalamin with a cobalt-carbon(alkyl) bond whose structure had been determined, until the present work, and so much of the data on alkylcobalt complexes comes from model systems.

Cobalamins are good subjects for X-ray crystal studies provided they are stable enough to be crystallised. The presence of the relatively heavy cobalt atom, which scatters X-rays much more strongly than do C, N or O atoms, means that the positions of the cobalt atoms in the unit cell can be obtained quite easily from the X-ray diffraction

data. The relative phases of all the reflections can then be computed by assuming that the structure contains only a cobalt atom, and considering the other atoms as small perturbations in the structure. Their positions are revealed by computing the electron density maps using first the relative phases calculated from the cobalt atom, and then successively more of the structure. The data are then refined using a least squares programme.

Other methods can be used, particularly when the structure does not contain a heavy atom, but the above method was used in the present work.

5.2 Crystal structures of cobalamins

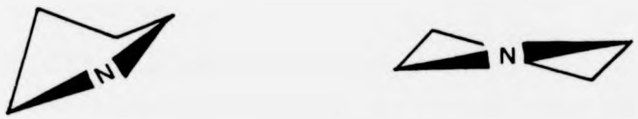
The X-ray crystallography of cobalamins and related systems has been reviewed by Glusker[24]. The crystal structures of cobalamins show considerable similarities which are briefly described here.

The cobalt atom shows distorted octahedral coordination. This is because the corrin ring is not planar, due to the direct link between rings A and D, and therefore the four equatorial nitrogen atoms cannot lie in a plane. The cobalt atom tends to keep the ring planar, as shown by the increased puckering in the cobalt-free corrin, but it cannot completely overcome the 'wave-like' structure of the corrin. Furthermore, the cobalt does not usually lie in the best plane through the equatorial nitrogen atoms, but projects towards one of the axial ligands, usually, it seems, to relieve steric strain. The axial Co-N bond length is in general longer than the average equatorial distance (in the coenzyme, $Co\text{-}N_{ax}$ = 2.24 Å, compared to $Co\text{-}N_{eq}$ = 1.90 Å (average)). The axial Co-C bond length is 2.03 (6) Å for adenosylcobalamin[33], compared to 1.92 Å for the Co-CN bond in cyanocobalamin. The Co-C-C angle has the unusually high value for

a tetrahedral carbon of 125 ± 3 °. The conformation adopted by the adenosyl ligand in the coenzyme is further discussed in section 5.4, where it is compared with the structures of the alkylcobalamins determined in this work.

The conformation of the corrin does not vary much between the cobalamins studied. The A and D rings take up an envelope-type conformation, while the B and C rings have the C2 (twist) geometry (figure 5.0). This puts the propionamido

Figure 5.0



"envelope" "twist"

and acetamido groups axial, on each ring except D, where they are equatorial. Thus, the acetamido groups on rings A and B point 'up' on the β-face of the corrin, while the propionamido groups on rings A, B and C point 'down' on the α-side. In addition, the methyl groups on rings C and D are also axial, on the β-face, while the methyl group on C1 (ring A) is axial and points 'down'. These 'sentinel groups' on each face of the corrin define quite rigidly the conformations available to the axial ligands, and the direction of attack of reagents approaching the cobalt atom (as discussed in Chapter 3).

The nucleotide loop also shows quite a similar conformation in all the cobalamins. The 5,6-dimethylbenzimidazole base lies in the least hindered channel of the α-side of the corrin, that is, parallel

to the C5 - C15 axis, with the 5,6-dimethyl groups nearer to C15. The Co-N-C bond angles are not equal (Co-N-B2 = 122.7 °, Co-N-B9 = 131.9 ° for the coenzyme) showing that the base bends down to minimise interactions with the corrin. This asymmetry is found in some other cobalt complexes as well. The corrin also shows a slight folding away from the axial base, about the Co-C10 axis (14 - 22 ° when the lower substituent is 5,6-dimethylbenzimidazole, less when it is CN). The extent of folding also depends on the size of the upper ligand, with larger ligands allowing a smaller folding, for steric reasons.

5.3 Crystal structures of B_{12} model compounds

Most of the information regarding the crystal structures of alkylcobalt species has come from cobaloximes and other B_{12} model complexes, since the only alkylcobalamin whose crystal structure was known, was adenosylcobalamin.

Studies of *trans*-effects in cobalt complexes[24] suggested the correlation of bond length with reactivity. Randaccio and Marzilli[197,198,24] studied a range of cobaloximes, and showed that for the same axial phosphine ligand, the Co-P bond length increases with increasing electron-donating ability of the ligand *trans* to it. Similarly in the Schiff base complex Co(SALOPH)(py)R, the Co-N(pyridine) bond length is 2.215(4) Å when R = ethyl, but is 2.098(4) Å when R = CH_2CN.

This *trans* ligand also affects the Co-C bond. For instance, replacing pyridine in isopropyl(pyridine)cobaloxime with triphenylphosphine causes an increase in Co-C bond length from 2.08 to 2.22(2) Å, although this may be due to a combination of steric and electronic factors[198]. This suggests that if the benzimidazole base in

the coenzyme is displaced when the cobalamin binds to a protein, and is replaced by another ligand, the properties of the Co-C bond could be altered.

The conformation of alkyl ligands in cobaloximes has also been studied[199-201], and the Co-C bond lengths have been measured. These steadily increase as the number and size of the substituents on Cα increases (Table 5.1). An advantage of using cobaloximes is that secondary, or even tertiary, alkylcobalt complexes can be prepared and crystallised, while the corresponding alkylcobalamins are very unstable.

Table 5.1

$[Co(DH)_2LR]$	Co-C Å	Co-C-C
R		
Me	2.009(7)	
CH_2Me	2.06(1)	122 °
$CHMe_2$	2.08	113-118 °
$C[CH_2]_3$ (adamantyl)	2.154(5)	108-114 °
CH_2CMe_3	2.06(6)	130 °

While Co-C bond lengths in cobaloximes are similar to those in cobalamins, the axial Co-N distance is shorter in the cobaloximes, suggesting a slightly greater positive charge on the cobalt atom than for cobalamins.

The Co-Cα-Cβ angle can also show an increase with increased steric interactions between the dimethylglyoximato ligands and the alkyl group (Table 5.1). In the case of the more rigid adamantyl structure, the steric strain is almost entirely relieved by lengthening of the Co-C bond.

The equatorial ligands of cobaloximes can fold relative to one another, away from the most bulky ligand, in the same way that cobalamins fold about the Co-C_{10} axis.

5.4 (*R*)- and (*S*)-2,3-Dihydroxypropylcobalamin

The crystal structures of these diastereoisomeric alkylcobalamins were determined, in order to compare the conformations of their alkyl ligands with that of adenosylcobalamin, to ascertain whether the Co-C bond length of 2.03(6) Å and the Co-Cα-Cβ angle of 125 (3) ° found in the coenzyme[33] are typical or atypical values, and whether there was any evidence for carbenoid or agostic[202] bonding at C1 as has been suggested[82].

The methods used for obtaining the crystals and determining the single crystal structures are given in the experimental section (5.5). We are grateful for the help of Dr. N.W. Alcock (Warwick University) in obtaining these structures.

Two views of each structure are given in Figures 5.1 - 5.4. The cobalamin part of each structure is quite similar to that of adenosylcobalamin (Fig. 5.5, also described in section 5.2). The alkyl ligands of the molecules do, however, show interesting differences. The cobalt-carbon bond lengths are not significantly different from that of the coenzyme, the bond length in the *R*-isomer being slightly shorter (2.002 (23) Å) and in the *S*-isomer slightly longer (2.079(30) Å). The Co-Cα-Cβ angles are both smaller than that of the coenzyme (*R*, 119.6 ± 1.7 °; *S*, 113.6 ± 2.1 °)(c.f. 125 ± 3 ° for the coenzyme).

In the coenzyme, the alkyl ligand is orientated so that the ribose ring lies in the channel between rings C and D, approximately

Figure 5.1 X-ray crystal structure of (R)- dihydroxypropylcobalamin
View from A-D side

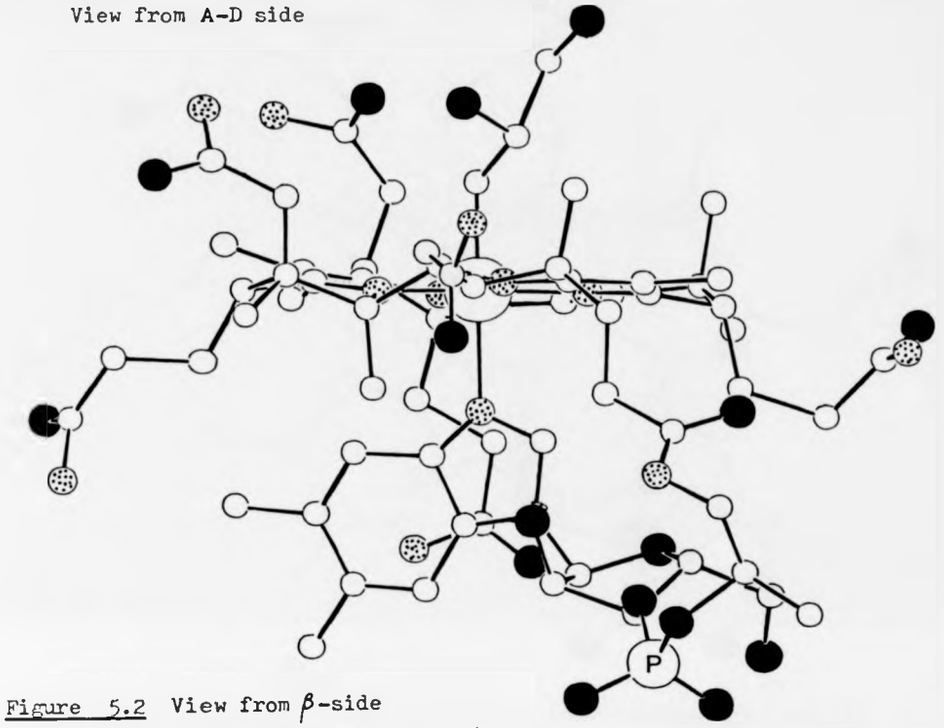


Figure 5.2 View from β-side
(nucleotide loop omitted for clarity)

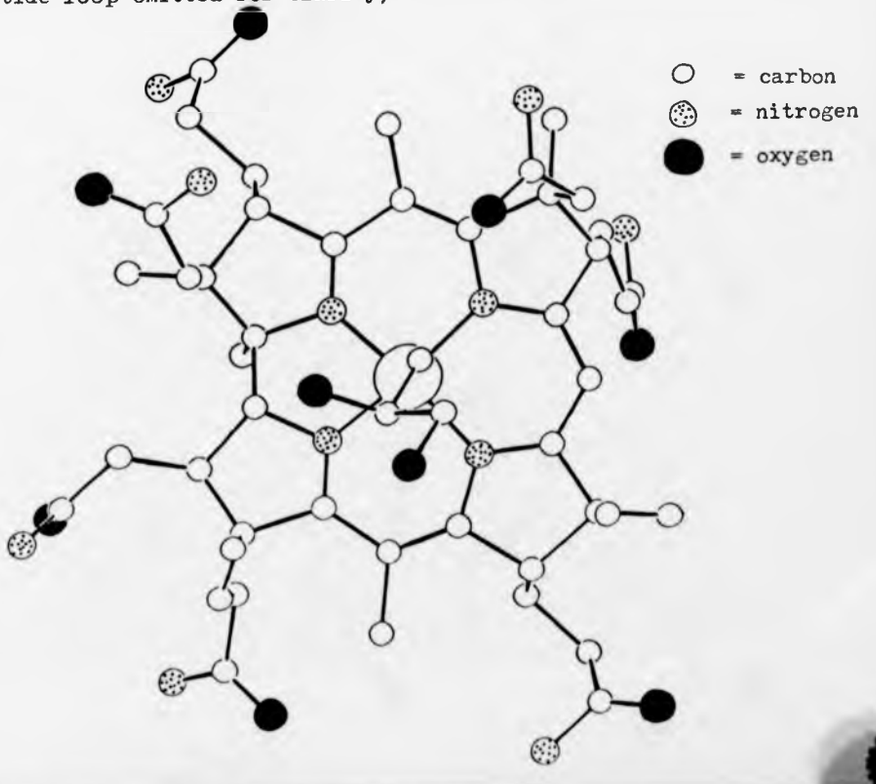


Figure 5.3 X-ray crystal structure of (S)-dihydroxypropylcobalamin

View from A-D side

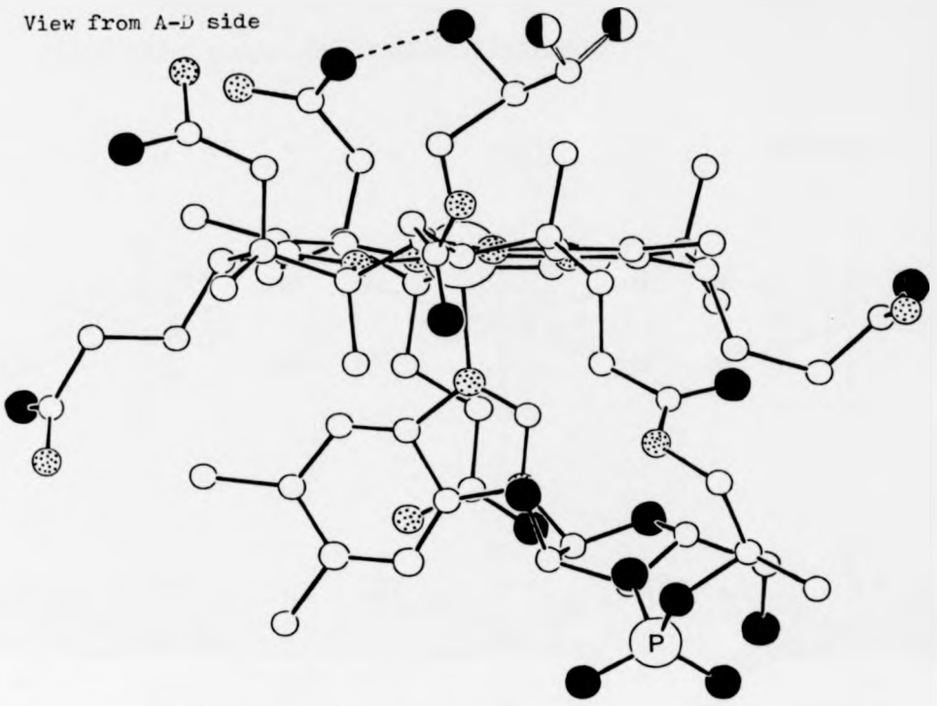
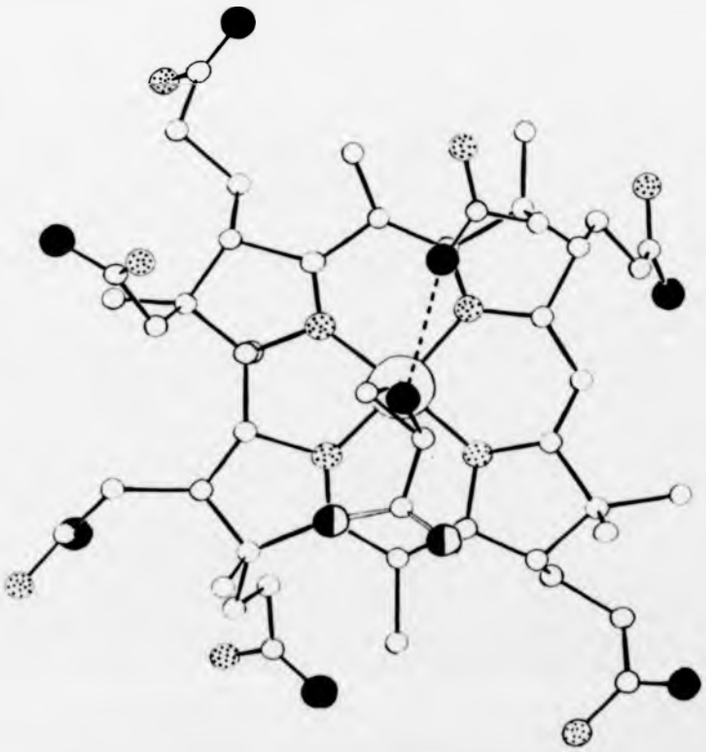


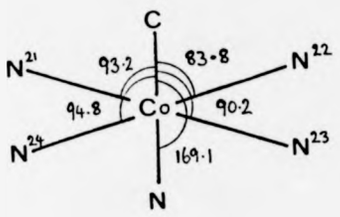
Figure 5.4 View from β-side (nucleotide loop omitted for clarity)

---- H- bond

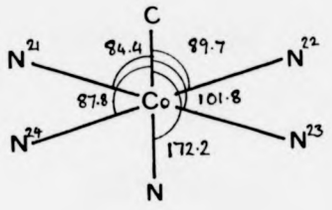


Coordination around the central cobalt atom

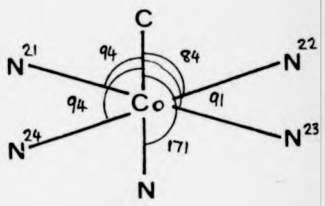
(R)

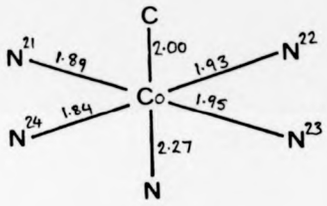


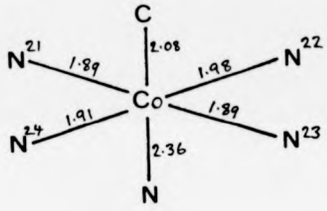
(S)

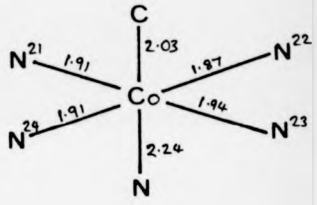


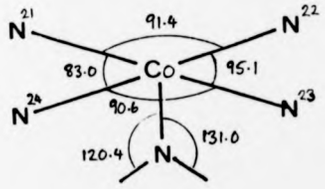
Adenosyl

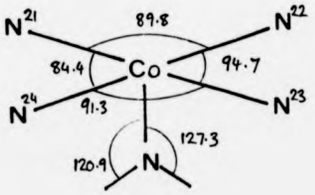


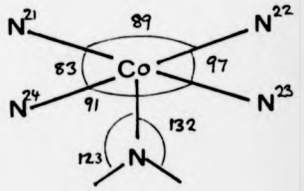








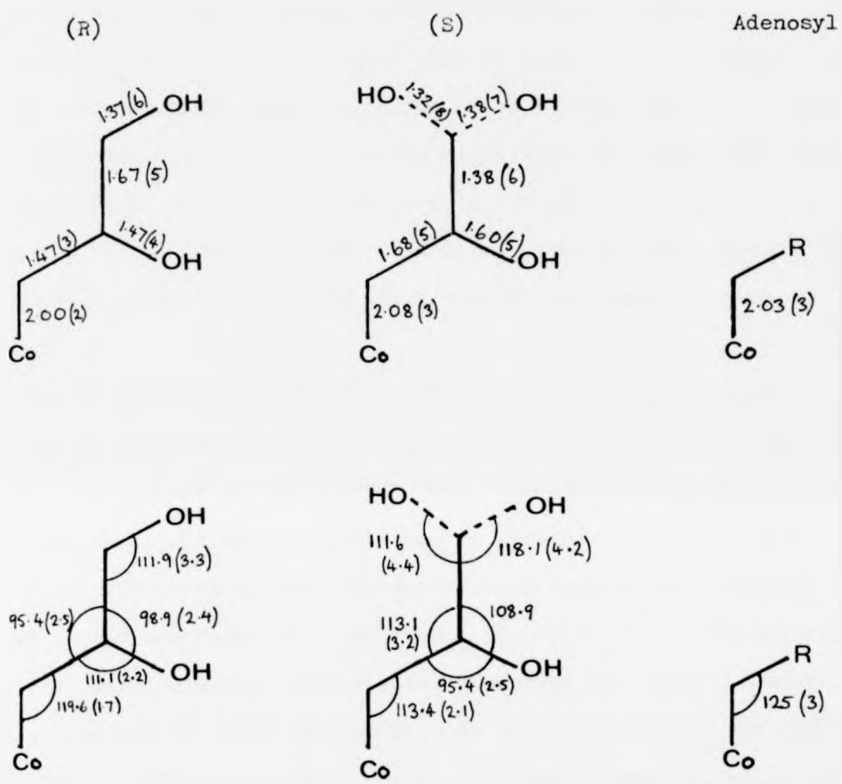




approx e.s.d.

.02	.03	.02 Å
0.9	1.2	1.5 °

Bond lengths (Å) and angles (°) in the alkyl ligands



e.s.d. in parentheses

perpendicular to the corrin. This is the least hindered channel on the β-face of the corrin, being flanked by two methyl groups, rather than by a methyl and an acetamido, or by two acetamido groups (section 5.2). The adenine base is thus positioned over ring C, approximately parallel to the plane of the corrin. It has been suggested that steric interactions between the corrin and the adenosyl ligand give rise to the large Co-Cα-Cβ angle, and this is supported by the two additional structures reported here, which have less sterically demanding alkyl groups, and smaller Co-Cα-Cβ angles.

The *S*-isomer (which has the corresponding chirality to the coenzyme at Cβ) uses the C-D channel for its $-CH_2OH$ group. This group is disordered in the crystal, with the hydroxyl group occupying two sites in approximately equal amounts (Figures 5.3, 5.4). The Cβ -OH is positioned so that it can hydrogen bond to the carbonyl oxygen on the acetamido group on ring B (O---O 2.76 Å). This latter group is correspondingly swung inwards towards the cobalt, compared to its position in other cobalamins. As in the coenzyme, the hydrogen on Cβ points down towards the corrin, for steric reasons, but in the coenzyme, Cα-Cβ lies nearly over the Co-N24 bond (torsional angle Ø = 16 °) whereas for the *S*-isomer Cα-Cβ is over Co-N23 (Ø = 10.7 °). The *R*-isomer (Figures 5.1, 5.2) has its Cα-Cβ above Co-N24 (Ø = 19.1 °) and accommodates its substituents at Cβ by placing the H atom in the channel between rings C and D, and the CH_2OH above this channel, which brings the hydroxyl group very close to C19-H. The effect of this proximity on the ^1H NMR chemical shift of C19-H in (*R*)-2-hydroxyalkylcobalamins is discussed in Chapter 4. A similar hydrogen bond to that observed for the *S*-isomer is not formed, because this would require the CH_2OH group to point down at ring C. Presumably the steric

crowding on the A-D side of the corrin prevents the formation of a hydrogen bond to the acetamido group on ring A, by preventing further rotation about the Co-Cα bond.

The increase in Co-Cα-Cβ angle from the *S*-isomer to the *R*-isomer, and from the *R*-isomer to the coenzyme, can be explained in terms of steric factors. The arrangement described for the *S*-isomer produces no severe steric interactions, and moreover profits from an intramolecular hydrogen bond. It therefore has almost a 'normal' tetrahedral angle. The *R*-isomer has no such hydrogen bond, but has close contact between Cβ-OH and C19-H, which causes an increase in the Co-Cα-Cβ angle to 119.6 °. In adenosylcobalamin, the steric interactions are minimised by placing the ribose in the C-D channel. This places the adenine over ring C, and the steric interactions are relieved by increasing the Co-Cα-Cβ angle to 125 °.

It seems, therefore, that in this series of compounds, steric strain is relieved by increasing the Co-Cα-Cβ angle, rather than by lengthening the Co-C bond. No special bonding need be invoked to explain the large angle found in adenosylcobalamin, instead, these observations provide evidence that the alkyl ligand in the coenzyme is under severe steric strain, and so presumably little activation by the protein is required to homolyse the Co-C bond in the enzymic reaction.

Recently, Finke and Hay[203] measured the bond dissociation energy of the Co-C bond in adenosylcobalamin (132 ± 5 kJ mol^{-1}). They estimated $\Delta G^{\dagger}$ (37 °) for the homolysis of this bond to be 124 kJ mol^{-1}, and point out that the rate of the propanediol dehydratase reaction requires a $\Delta G^{\dagger}$ of about 63 kJ mol^{-1}. Therefore

the enzyme must activate the Co-C bond to the extent of about 60 kJ mol^{-1} to account for the observed rate, corresponding to a rate increase of at least 10^{10} times. This required activation appears large, but could be produced by a relatively small conformational change in the corrin, initiated by a change in the conformation of the protein.

5.5 Experimental

5.5.1 Preparation of crystals

(*S*)-Dihydroxypropylcobalamin: This compound was crystallised in the usual way (Chapter 3) from a concentrated aqueous solution (~ 20 mg/ml) by the addition of acetone until a faint cloudiness

persisted. On leaving overnight, red, needle-shaped crystals were deposited. These were recovered by filtration, washed with acetone, and air dried.

(*R*)-Dihydroxypropylcobalamin: The above method gave crystals that were too small for single crystal studies. A number of solvent systems were tried, and suitable crystals were finally obtained from an aqueous bis(methoxyethyl)ether (diglyme) solution, after slow evaporation of the water by passing a slow stream of nitrogen over the solution. The crystals were recovered by filtration, and were washed with aqueous acetone (9 : 1, acetone : water), followed by acetone and dry ether, and were dried in a stream of dry nitrogen.

5.5.2 Crystal data (Data from Dr. N.W. Alcock)

(*R*-form, from diglyme-water): $C_{65}H_{95}Co\,N_{13}O_{16}P$, *ca.* 15 H_2O
M = 1404.5 (excluding water), orthorhombic, space group $P2_12_12_1$ with
a = 24.779(7), b = 21.008(7), c = 16.049(5) Å.
U = 8354(5) Å^3, Z = 4, Mo-K_α radiation (λ = 0.71069 Å) μ(Mo-K_α) = 2.83 cm^{-1}.

R = 0.085 for 2266 reflections [$I/\sigma(I) > 3.0$] collected by a syntex P2, four-circle diffractometer and corrected for Lorentz and polarisation effects.

(*S*-isomer, from acetone-water): orthorhombic, space group $P2_12_12_1$ with a = 24.43(3), b = 21.13(3), c = 16.08(2) Å,
U = 8299(18) Å^3, Z = 4, Mo-K_α radiation (λ = 0.71069 Å),
μ(Mo-K_α) = 2.83 cm^{-1}.

R = 0.151 for 2299 reflections [$I/\sigma(I) > 3.0$] collected as for the *R*-isomer. In contrast to the *R*-form, which was stable and diffracted more strongly, the *S*-isomer diffracted weakly and decayed during data collection.

For both isomers, hydrogen atoms were placed in calculated positions without refinement.

CHAPTER 6

PHOTOLYSIS AND THERMOLYSIS OF DIHYDROXYALKYLCOBALAMINS

CHAPTER 6

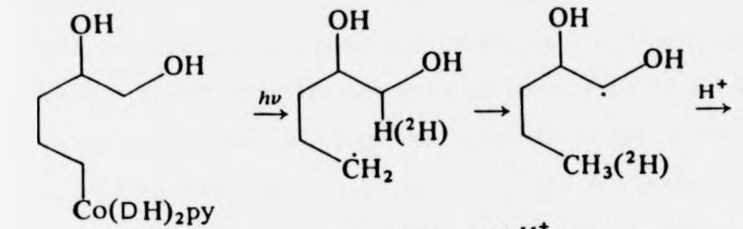
PHOTOLYSIS AND THERMOLYSIS OF DIHYDROXYALKYLCOBALAMINS

6.1 Diol dehydratase model systems

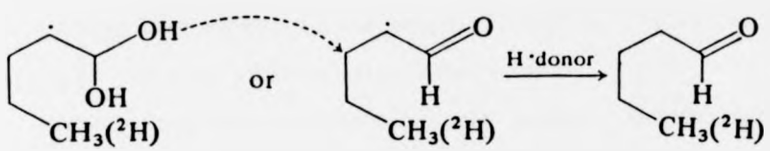
The model for the vitamin-B_{12}-dependent diol-dehydratase reaction presented in this work, depends on the homolysis of the cobalt-carbon bond in the model compounds, to give an alkyl radical ($R\cdot$) and a cobalt(II) species. In the enzymic reaction, the first step is generally agreed to be the homolysis of the cobalt-carbon bond of adenosylcobalamin. The resulting 5'-deoxyadenosine radical then abstracts a hydrogen atom from the substrate diol, to give a substrate-derived radical. The evidence for these steps is discussed in Chapter 1. The substrate radical then rearranges in some way to give a radical resembling the product, which repossesses the hydrogen atom from 5'-deoxyadenosine to give the product.

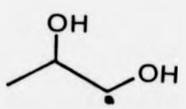
It was hoped that dihydroxyalkylcobalamins would act as models for the first two steps in this sequence. That is, homolysis of the cobalt-carbon bond would lead to an alkyl radical, which in turn would undergo an intramolecular hydrogen-transfer to give an isomeric radical, resembling the intermediate postulated in the enzymic reaction (Scheme 6.1). Thus one end of the alkyl ligand is acting as the adenosyl group, and the other end as the substrate diol. Formation of a product-type group is possible evidence of the intermediacy of the radical (58) in the enzymic reaction, and can give some indication of the conditions which are required to give the product-like radical. Some questions remain, for instance, whether electron transfer occurs, or whether there is participation by cobalt in the rearrangement. The model and theoretical studies relevant to these questions have been summarised in Chapter 1.

Scheme 6.1 Formation of pentanal[56]



$$\dot{C}o(DH)_2py \xrightarrow{aq\ H^+} aqCo(II) + pyH^+ + DH_2$$





Golding *et al.*[31] showed that a [1,5]-hydrogen shift occurred when 4,5-dihydroxypentylcobaloxime was photolysed, and pentanal was produced. A similar shift occurred on photolysis of 4,5-dihydroxycyclooctylcobaloxime, which produced cyclooctanone in 40 % yield[56]. These studies show that for cobaloximes, the production of a radical derived from a 1,2-diol is possible by this means, and that, once formed, such a radical gives, under acidic conditions, a product resembling that formed in the enzymic reaction. It was of interest to study the same systems using cobalamins.

The enzymic reaction is not light induced; the labilisation of the cobalt-carbon bond in adenosylcobalamin is probably induced by a conformational change in the corrin, which increases the already considerable steric interactions between the adenosyl group and the corrin[35]. This may in turn be initiated by a change in the protein conformation, induced by substrate binding. This ensures that the reactive radical is produced only in the presence of substrate. In the model reactions, homolysis of the cobalt-carbon bond was produced by exposing the cobalamins to light or heat in aqueous solution. It was therefore important to determine the possible pathways for decomposition of alkylcobalamins under these conditions.

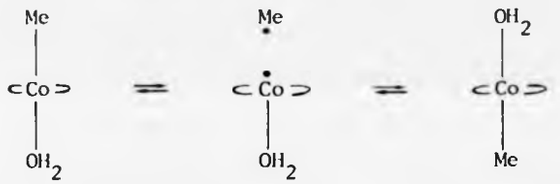
6.2.1 Previous studies

The photolysis of methylcobalamin has been most extensively studied[204,205]. Decomposition is quite slow in the absence of oxygen, and gives methane, ethane and cobalamin(II) as products. In the presence of air, it is approximately 1200 times faster, and gives formaldehyde, and aquocobalamin, with traces of methane, methanol and formic acid. In the presence of other radical scavengers, such

as thiols, quinones and certain alcohols, photodecomposition is also rapid, and leads to products consistent with the trapping of methyl radicals. Thus the photolysis of methylcobalamin in the presence of 2-propanol leads to the formation of cobalamin(II) and pinacol, as well as methane, suggesting that the methyl radical abstracts the α-hydrogen from the alcohol[206].

In addition, photolysis of methylaquocorrinoids in the absence of air, leads to an exchange of the upper and lower ligands[207], (Equation 6.1).

Equation 6.1



The flash photolysis of methylcobalamin was studied by Endicott and Ferraudi[160], who showed that cobalamin(II) is the initial product, and is formed at the same rate, in the presence or absence of air. Recombination of the methyl radical with the cobalt(II) species is, however, very fast ($k \sim 1.5 \times 10^9$ mol^{-1} sec^{-1}) so in the absence of other reagents, even prolonged exposure to light does not lead to decomposition. All these findings are consistent with the hypothesis that homolysis of the cobalt-carbon bond of methylcobalamin occurs.

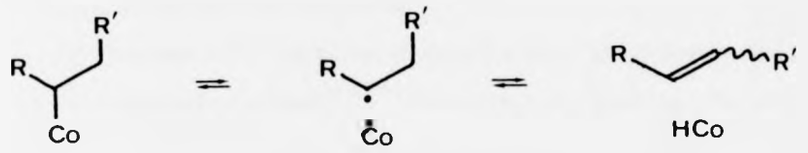
The thermal decomposition of a number of cobalamins with larger alkyl ligands has been studied by Chemaly and Pratt[155], and by Grate and Schrauzer[118]. They show that, in general, more sterically compressed alkylcobalamins are more labile, and that base-off cobalamins are more stable than base-on ones. This effect has

been discussed in Chapter 3, that is, the dissociation of the 5,6-dimethylbenzimidazole base allows the steric compression around the alkyl ligand to be relieved. They also found that two pathways of decomposition compete, in most cases. One pathway leads to the formation of olefins by β-elimination of hydrogen, and one leads to products derived from the radicals produced on homolysis of the cobalt-carbon bond. For instance, the decomposition of ethyl cobalamin leads to ethane and ethene[208]. Halpern *et al.*[52] suggested that both these pathways could arise by the initial homolysis of the cobalt-carbon bond, followed either by β-elimination between the cage-trapped radical and the cobalamin(II), to give the olefin, or by escape of the alkyl radical, which abstracts a hydrogen atom from some other molecule to give the alkane, Equation 6.2a. In the example above the proportion of ethane to ethene is increased on the addition of mercaptoethanol, and the rate of decomposition is increased by reagents such as O_2 and 2-propanol, suggesting that cobalt-carbon bond homolysis is indeed the first step.

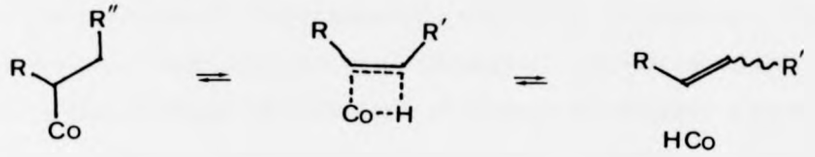
The rate of decomposition of strained secondary alkylcobalamins, such as isopropylcobalamin, is very rapid, and is independent of the presence or absence of oxygen, and increases as the alkyl ligand is further substituted on the β-carbons[118]. The insensitivity to oxygen or other reagents led to the conclusion[118] that β-elimination occurs *via* a four centre transition state as in Equation 6.2b.

These observations suggest that either a stepwise or a concerted pathway for β-elimination can occur. The stepwise pathway may be more important in the photolytic decomposition of alkylcobalamins, while the concerted mechanism may occur when the cobalamins are thermolysed.

Equation 6.2a.



Equation 6.2b.



The nature of the cobalt-containing species produced by β-elimination is not clear. Schrauzer and Grate[118] suggest that it is a cobalt-hydride, hydridocobalamin. They show that it is the same as the species produced by the reduction of cobalamin(III) by zinc dust in acetic acid, by demonstrating that decomposition of isopropylcobalamin in the presence of methyl iodide leads to methylcobalamin in 95 % yield. They also studied the decomposition of cyclooctylcobalamin in DCl/D_2O, and found HD and D_2 in the gas phase, which is evidence for the initial formation of hydridocobalamin.

Pratt[35] however, suggests that the site of protonation is not the cobalt atom, but is instead perhaps C5, C10 or C15 on the corrin system. The ability of the C10-H to exchange with deuterium in acid solution is evidence that the corrin ring can be protonated.

The rate of photolytic decomposition of ethyl- and higher alkyl-cobalamins is increased by the presence of oxygen, suggesting that light induces the homolysis of the Co-C bond. In the absence of

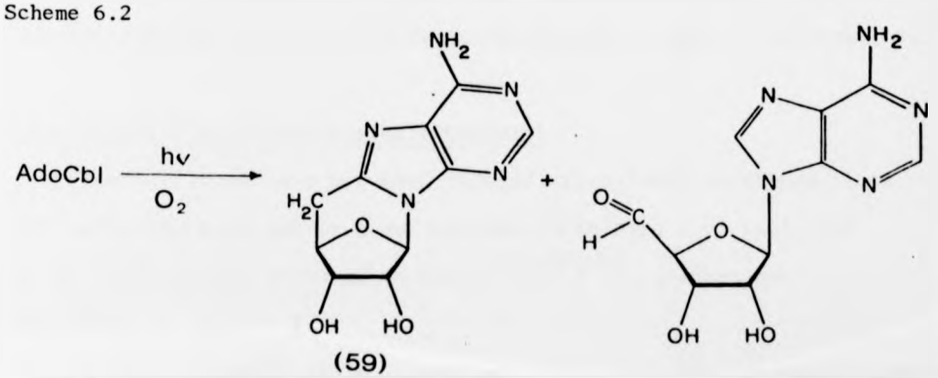
oxygen, olefins are the main products, while in the presence of oxygen, aliphatic aldehydes are produced[209,210].

In the case of cobalamins unable to undergo β-elimination, such as neopentylcobalamin[152,155], homolytic fission is the only pathway for decomposition. Neopentylcobalamin is stable in neutral, anaerobic solution in the dark, but decomposes rapidly in the presence of oxygen[152] ($t_{\frac{1}{2}}$ ~ 60 min), and of other radical scavengers. In the presence of mercaptoethanol, neopentane is produced. The oxygen- and light-sensitivity is decreased in acidic solution, where the cobalamin is base-off. It therefore provides a model for the labilisation of the Co-C bond by steric distortion. We planned to use a neopentyl-type cobalamin to model the homolysis of the Co-C bond in adenosylcobalamin.

6.2.2 Photolysis of adenosylcobalamin

The photolysis of adenosylcobalamin proceeds *via* homolytic fission of the cobalt-carbon bond. In anaerobic conditions adenosylcobalamin decomposes rapidly ($t_{\frac{1}{2}}$ ~ 3 min, as opposed to methylcobalamin $t_{\frac{1}{2}}$ ~ 20 h), to give 5'-deoxy-8,5'-cycloadenosine (59). In air, photolysis is slightly faster ($t_{\frac{1}{2}}$ ~ 1 min), and adenosine-5'-aldehyde is produced as well as (59)[208].

Scheme 6.2



(59)

With thiols and 2-propanol, 5'-deoxyadenosine is produced. Flash photolysis[179] gives cobalamin(II) at a rate unaffected by the presence of oxygen or 2-propanol, and the rate of recombination of the cage-trapped radical pair is similar to that of methylcobalamin.

It seems that β-elimination cannot occur for steric reasons, and so the initially formed radical can decompose only by cyclisation on to the 8-position of the adenine base. This cyclisation is presumably prevented in the enzyme by the protein holding the coenzyme in a particular conformation.

A model for diol dehydratase is obtained by the photolysis of adenosylcobalamin in the presence of substrate, but this is only successful in situations where cyclisation to (59) is prevented. For instance, aerobic photolysis of 8-methoxyadenosylcobalamin causes conversion of ethanediol, ethanolamine and ethoxyethylamine into acetaldehyde[99]. Similarly, if adenosylcobalamin is photolysed with ethanediol and a thiol such as dihydrolipoamide, acetaldehyde is produced, but not in the absence of the thiol[98].

Adenosylcobalamin is quite stable to heat in aqueous solution. Like methylcobalamin, it can be heated at 94 ° for up to 5 h without changing the ^{13}C NMR spectrum. In contrast ethylcobalamin gives extensively broadened lines, demonstrating the presence of cobalamin(II), after only 2 min at this temperature[211]. This demonstrates the stability of adenosylcobalamin towards β-elimination.

6.2.3 Decomposition of dihydroxyalkylcobalamins

These systems have not been studied extensively, although there has been wide interest in model systems containing a vicinal diol on an alkyl ligand attached to cobalt[31,100,102], which are described in Chapter 1.

An early study[212] of the photolysis of 2,3-dihydroxypropylcobalamin

suggested that glycerol was produced in the absence of oxygen, and glyceraldehyde and glyceric acid in the presence of oxygen. This has been questioned[209], on the basis of the known photodecomposition of β-hydroxypropylcobaloximes to give acetone[213], and β-hydroxyisopropyl(pyridine)cobaloxime to give alkyl alcohol and propanal[214,215]. Under acidic conditions, 2-hydroxyethylcobalamin[216] decomposes to give ethene and hydroxocobalamin, possibly *via* a π-complex. In the present study, we showed that (*R*)- and (*S*)-dihydroxypropylcobalamin do in fact decompose to give allyl alcohol and hydroxyacetone (section 6.4). The original study[212] relied on paper chromatography and the reaction of periodate to identify the products, while we were able to characterise them fully by high field ^{1}H NMR spectroscopy, and by gas chromatography.

In the present work, the cobalamins produced by the methods described in Chapter 3, were photolysed and thermolysed in aqueous solution. Both the photolysis and thermolysis were studied, in case there were differences in the mode of cobalt-carbon bond breakage under these conditions. The effect of the presence or absence of oxygen was studied, and, in some cases, the effects of pH. The products were identified by high-field ^{1}H NMR spectroscopy, and, in some cases, by gas chromatography and derivatisation. Comparison of the products with authentic samples was employed where possible.

6.3.1 General experimental procedures

The alkylcobalamin (∿ 7 mg, ∿ 5 x 10^{-3} mmol) was dissolved in D_2O (0.5 ml) with TSS as an internal standard. The solution, in a precision-quality 5 mm NMR tube, was at all times protected from light.

a. Anaerobic experiments: The solution, in an NMR tube fitted with a B10 cone, was carefully degassed, either by repeated freeze-pump-thaw cycles under nitrogen, or by the passage of argon. The NMR tube was then sealed in a flame.

b. Aerobic experiments: The solution was saturated with oxygen by the slow bubbling of air or oxygen, then the tube was capped.

The ^{1}H NMR spectrum of the solution was recorded immediately before the decomposition was started.

c. Photolysis: This was accomplished by irradiating the solution with visible light from a 100 W tungsten-filament bulb, at a distance of $\sim$ 20 cm. The NMR tube containing the solution was kept at 20 ± 5 ° in a pyrex water bath. Photolysis was allowed to proceed for 6 - 10 h, and in most cases, the cobalamin solution had become brown in colour, signifying the formation of cobalamin(II).

d. Thermolysis: The thermolysis experiments involved heating the solution at 90 ° in a thermostatted oil bath, the tube being protected from light with aluminium foil. The solutions were heated for 6 - 10 h, and, like the photolysed solutions, they became dark brown in colour.

6.3.2 Identification of products

6.3.2a. ^{1}H NMR: The initially produced, dark brown, solutions gave poorly resolved ^{1}H NMR spectra, the lines being broadened by the paramagnetic cobalamin(II), so oxygen was bubbled through the solution to reoxidise the cobalt(II) to cobalt(III). In some cases, the excess of oxygen

was removed by the passage of nitrogen, in order to improve the spectral resolution. The ^1H NMR spectrum was then recorded, after which, the pH of the solution was measured.

6.3.2b. Dinitrophenylhydrazones

The presence of carbonyl-containing compounds was investigated by the addition of dinitrophenylhydrazine (DNP) solution (0.4 % in 1 M H_2SO_4, 0.2 ml) to the sample[56]. After 2 - 3 h at room temperature, the resulting dinitrophenylhydrazones were extracted out of the aqueous solution with dichloromethane, and analysed by t.l.c. (silica gel, CH_2Cl_2). Authentic DNP derivatives of suspected products were run as standards. Unreacted DNP could be removed from the mixture by passage down a short silica gel column. The ^1H NMR spectra of the remaining DNP derivatives were recorded, but in general the amounts of these species were too low to give identifiable signals.

6.3.2c. Gas chromatography

In the case of the 2,3-dihydroxypropylcobalamins, the identity of the products was confirmed by gas chromatography, (column: 0.1 % SP1000 on Carbowax; chromatograph: PE 204). Samples of glycerol, glyceraldehyde, 3-hydroxypropanal, propanal, propane-1,2-diol, propane-1,3-diol, hydroxyacetone and allyl alcohol were all resolved on this system, using a temperature programme. Only the last two compounds were found among the reaction products from the above cobalamins, and co-injection of authentic samples with the reaction mixture gave an increase in the intensity of each peak in turn.

6.4 Results and discussion

The photolyses and thermolyses of the cobalamins were carried out according to the methods outlined in section 6.3.1.

6.4.1 The cobalamin products

It was not possible to obtain much information about the initially formed cobalamin species, as the solutions always contained enough paramagnetic cobalamin(II) to give poorly resolved spectra. Therefore, oxygen was blown through the solutions to oxidise the cobalamin(II) to cobalamin(III). Cobalamin(II) was found even in the 'aerobic' experiments, showing that the dissolved oxygen in the solution was used up and was not replenished at a rapid enough rate from the atmosphere.

The ^{1}H NMR spectra of the oxygen-treated reaction mixtures usually showed more than one cobalamin species. Hydroxo- or aquocobalamin (depending on pH) was the major product (> 60 %) in all cases except for the aerobic thermolysis of dihydroxyhexylcobalamin. The other species were not identified, as the only peaks assignable to a particular cobalamin were those downfield of the HDO signal, corresponding to C10-H, R1-H, B2-H, B4-H and B7-H. No starting material was found. There were two distinct sets of resonances, designated A and B in Table 6.2, which shows the distribution of products in the various experiments. A was found in all the experiments except for those using dihydroxybutylcobalamin, where, instead, B was found. The t.l.c. of the reaction mixtures showed that hydroxocobalamin was present, and also a fast running brownish spot ($R_f \sim 0.6$, cellulose solvent A).

In general, the least hydroxocobalamin was formed in the aerobic thermolysis of cobalamins, and the most in the photolysis experiments,

but beyond that, no discernible pattern emerged.

Table 6.1: Chemical shifts (δ, p.p.m.) of low field signals of the various cobalamins in Table 6.

	OHCbl	A	B
C10	6.08 (variable)	6.05	6.06
R1	6.23	6.29	6.26
B7	6.48	6.41	6.27
B2	6.67	6.81	6.98
B4	7.05	7.22	7.18

6.4.2 Organic products

The signals in the ^1H NMR spectra which did not correspond to cobalamin protons, were assumed to have arisen from the alkyl ligands. They were often recognisable as they had narrower lines than the cobalamin resonances, but the region upfield of ∿ δ 2.5 was not very useful, as so many overlapping resonances occur in that region. The products are listed in Tables 6.3 to 6.7 and examples of the spectra are shown.

Key to Tables 6.3 to 6.7

The percentages were estimated from the integrals of the signals corresponding to the organic products, relative to the integrals of cobalamin resonances. Unidentified resonances were treated as though they consisted of one proton, and the percentage is given relative to the integral of one cobalamin proton. The values are accurate to ± 10 %.

Table 6.2: Distribution of cobalamin products

Alkyl group	Conditions	OHCbl	A	B	Others	H/D exchange
(*S*)-dihydroxypropyl	h ν O_2	95	5		< 5	
	h ν no O_2	100				
	Δ O_2	60	20		20	B2
	Δ no O_2	80	20			B2
(*R*)-dihydroxypropyl	h ν O_2	95	5			
	h ν no O_2	90	10			
	Δ O_2	70	20		10	B2
	Δ no O_2	80	15		5	B2
(*R* + *S*)-dihydroxybutyl	h ν O_2	80		20		
	h ν no O_2	65		35		
	Δ O_2	-		-		
	Δ no O_2	90		10		B2, C10
(*R* + *S*)-dihydroxypentyl	h ν O_2	100				
	h ν no O_2	95	5			
	Δ O_2	50	40		10	B2
	Δ no O_2	70	30			B2
(*R* + *S*)-dihydroxyhexyl	h ν O_2	66	33			
	h ν no O_2	90	10			
	Δ O_2	30	60		10	
	Δ no O_2	75	25			C10, B2

^{1}H NMR signals quoted are those of unidentified organic products. The identified products are given letters: A (hydroxyacetone), B (allyl alcohol), C (butene-3,4-diol), D (pent-1-ene-4,5-diol), E (pent-2-ene-4,5-diol) and F (hex-1-ene-5,6-diol), and their ^{1}H NMR data are listed in Table 6.8.

Other evidence: GC - the compound had an identical retention time to that of an authentic sample. DNP - the dinitrophenylhydrazone of the compound had an identical R_f to that of an authentic sample.

Table 6.3: (*S*)-2,3-Dihydroxypropylcobalamin decomposition products

Conditions	Organic products	^{1}H NMR	Other evidence
aerobic photolysis p^2H 8.2 (air)	hydroxyacetone 70 % (80 % D-labelled)	A 3.52 (d, J = 5 Hz) < 10 % 5.06 (t, J = 5 Hz) < 5 % 5.40 (s) < 5 % 5.47 (s) < 5 %	GC, DNP
anaerobic photolysis p^2H 8.2	hydroxyacetone 63 % (75 % D-labelled)	A	GC, DNP
	allyl alcohol 37 %	B	GC
anaerobic photolysis p^2H 4 (KCl/DCl)	hydroxyacetone 75 % (78 % D-labelled)	A	GC, DNP
	allyl alcohol 25 %	B	GC
aerobic thermolysis I p^2H 7.7 (air)	hydroxyacetone 69 % (90 % D-labelled)	A	GC, DNP
	allyl alcohol 31 %	B	GC
aerobic thermolysis II (oxygen)	hydroxyacetone 16 % (90 % D-labelled)	A 5.04 (40 %, t, J = 5 Hz) 3.51 (20 %, d, J = 5 Hz) 4.40 (15 %, s)	
anaerobic thermolysis pH 8	hydroxyacetone < 5 %	(A)	
	allyl alcohol 50 %	B 3.51 (dd, J = 6, 10 Hz) + other signals ∿ 3.5-3.6	

Table 6.4: (*R*)-2,3-Dihydroxypropylcobalamin decomposition products

Conditions	Organic products	^{1}H NMR	Other evidence
aerobic photolysis p^{2}H	hydroxyacetone 43 % (84 % D-labelled)	A 3.51 (d, J = 5 Hz) 4.94 (d, J = 5 Hz) 5.05 (t, J = 5 Hz) 5.38 (s) 5.45 (s)	GC, DNP
anaerobic photolysis p^{2}H 7	hydroxyacetone 66 % (70 % D-labelled)	A	GC, DNP
	allyl alcohol 34 %	B	GC
aerobic thermolysis	hydroxyacetone 72 % (90 % D-labelled)	A	DNP
	allyl alcohol 13 %	B ~ 3.5 m 4.41 s	
anaerobic thermolysis	hydroxyacetone 20 % (70 % D-labelled)	A	GC, DNP
	allyl alcohol 75 %	B	GC

Table 6.5: (3*R* + 3*S*)-3,4-Dihydroxybutylcobalamin decomposition products

Conditions	Organic products	^{1}H NMR
aerobic photolysis p^2H 7.5	butene-3,4-diol 50 %	C 5.69 (dd, J = 6,4) 30 % 5.53 (d, J = 4) 15 % 4.5-4.6 (m) 30 %
anaerobic photolysis p^2H 8	butene-3,4-diol 100 %	C
aerobic thermolysis I (air)	butene-3,4-diol 65 % (possibly D-labelled)	C 4.37 (1 : 1 : 1t) < 5 % 5.24 (dd, J = 1.5, 11 Hz) < 5 % 5.33 (dd, J = 1.5, 17 Hz) < 5 % 5.85 (m) < 5 %
aerobic thermolysis II (oxygen)	butene-3,4-diol 30 %	C 5.53 (d, J = 4) 10 % 5.69 (dd, J = 6,4) 10 % 4.5-4.6 (m) 30 %
anaerobic thermolysis	butene-3,4-diol (20 % from olefinic integral 60 % from CH(OD)-CH_2(OD) ∴ possibly D-labelled on C1)	C 5.24 (dd, J = 1.5, 11 Hz) 10 % 5.33 (dd, J = 1.5, 17 Hz) 10 % 5.85 (m) 20 % 4.35 (t) 10 %

Table 6.6a: (4*S*)-4,5-Dihydroxypentylcobalamin decomposition products

Conditions	Organic products	^{1}H NMR
aerobic photolysis pH 7.1	pent-1-ene-4,5-diol 100 %	D
anaerobic thermolysis	pent-1-ene-4,5-diol 29 %	D
	(?) pent-2-ene-4,5-diol 71 %	E

Table 6.6b: (4*R* + 4*S*)-4,5-Dihydroxypentylcobalamin decomposition products

Conditions	Organic products	^{1}H NMR
aerobic photolysis (I) (air)	pent-1-ene-4,5-diol 20 % (? D-labelled on C1)	D
	(?) pent-2-ene-4,5-diol 70 %	E 5.35 (10 %, m)
aerobic photolysis II (oxygen)	pent-1-ene-4,5-diol ∿ 100 % (?D-labelled on C1)	D
anaerobic photolysis pH 8	pent-1-ene-4,5-diol 60 %	D
	(?) pent-2-ene-4,5-diol 40 %	E
anaerobic photolysis p^2H 3.4 ($^2H_4$-acetic acid)	(?) pent-2-ene-4,5-diol ∿ 100 %	E
aerobic thermolysis	pent-1-ene-4,5-diol 100 % (not labelled)	D
anaerobic thermolysis	pent-1-ene-4,5-diol 70 % (? D-labelled on C1)	D
	(?) pent-2-ene-4,5-diol 30 %	E

Table 6.7: (5*R* + 5*S*)-5,6-Dihydroxyhexylcobalamin decomposition products

Conditions	Organic products	^{1}H NMR
aerobic photolysis	hex-1-ene-5,6-diol 100 %	F
anaerobic photolysis	hex-1-ene-5,6-diol 20 %	F
	(?) hex-2-ene-5,6-diol 80 %	5.45 (1H, dt, J ∿ 15, 6, 6 Hz) 5.6 (1H, m) 3.47 (1H, dd, J = 7, 12) 3.51 (1H, dd, J = 8, 12) 3.7 (m?)
aerobic thermolysis	hex-1-ene-5,6-diol 100 %	F
anaerobic thermolysis	(?) hex-2-ene-5,6-diol (20 % ?)	5.45 (30 %, m) 5.6 (15 %, m) 3.47 (20 %, dd, J = 7, 12) 3.51 (10 %, dd, J = 8, 11)

Table 6.8: ^{1}H NMR data for products A-F in Tables 6.3-6.7 (D_2O, 400 MHz)

A	Hydroxyacetone*	δ 2.14 (3H, s, $-COCH_3$), 4.36 (2H, s, $-CO-CH_2-OD$)
	[3-^{2}H]-Hydroxyacetone	δ 2.12 (2H, 1 : 1 : 1t, J = 2.3 Hz, $-COCH_2D$), 4.36 (2H, s, $-CO-CH_2OD$)
B	Allyl alcohol*	δ 4.10 (2H, ddd, J = 5.3, 1.5, 1.5 Hz, $-CH_2OD$), 5.17 (1H, dddd, J_{cis} = 10.5, J_{gem} = 1.5, $J_{allylic}$ = 1.5, 1.5 Hz), 5.27 (1H, dddd, J_{trans} = 17.3, J_{gem} = 1.5, $J_{allylic}$ = 1.5, 1.5 Hz, $=CH_2$), 6.00 (1H, dddd, J = 10.6, 17.3, 5.3, 5.3 Hz, -CH =).
C	Butene-3,4-diol*	δ 3.52 (1H, dd, J = 11.6, 6.6 Hz, HCHOD), 3.60 (1H, dd, J = 11.6, 4.7 Hz, HCHOD), 4.20 (1H, m, -CH-OD), 5.25 (1H, ddd, J = 10.6, 1.4, 1.4 Hz), 5.33 (1H, ddd, J = 17.4, 1.5, 1.4 Hz, H_2C = C), 5.86 (1H, m, = CH-)
D	Pent-1-ene-4,5-diol*	δ 2.17, 2.30 (2H, AB portion of ABXM system, J_{AB} = 14.4, J_{AX} = 7.7, J_{BX} = 6.6, J_{AM} = 7.7, J_{BM} = 5.1, $J_{allylic}$ = 1.2, 1.2, 1.3, 1.3 Hz, = C-CH_2-C), 3.48, 3.59 (2H, AB portion of ABX system, J_{AB} = 11.7, J_{AX} = 6.81, J_{BX} = 4.0 Hz, $-CH_2OD$), 3.77 (1H, m, -CH(OD)-), 5.13 (2H, m, $=CH_2$), 5.85 (1H, dddd, J_{trans} = 17.2, J_{cis} = 10.3, J_{vic} = 7.7, 6.6 Hz, -CH =).

Table 6.8: contd.

E	(?) Pent-2-ene-4,5-diol	δ 3.51 (1H, dd, J = 7, 11 Hz, HCHOD), 3.55 (1H, dd, J = 5, 11 Hz, HCHOD), 4.14 (1H, m, -CH-OD), 5.48 (1H, dd, J = 15, 7 Hz, -CH=), 5.84 (1H, m, =CH-). (the methyl group was not located.)

Decoupling experiments showed that the signal at δ 5.48 is coupled to δ 4.14 (7 Hz) and 5.84 (11 Hz), and the signal at δ 4.14 is also coupled to δ 3.51 (7 Hz) and 3.55 (5 Hz).

F	Hex-1-ene-5,6-diol	δ 3.49 (1H, dd, J = 11.5, 7 Hz, -HCHOD), 3.59 (1H, dd, J = 11.5, 8 Hz, -HCHOD), ∿ 3.65 (1H, m, -CHOD), 5.02 (1H, dd, J ∿ 2, 11 Hz), 5.10 (1H, dd, J ∿ 2, 17 Hz, = CH_2), 5.91 (1H, m, -CH =) (the $-CH_2-$ $-CH_2-$ signals were not located).

* Authentic samples of these compounds were available or were prepared. The others were identified from the spectra of the reaction mixtures.

6.4.3 2,3-Dihydroxypropylcobalamins

Tables 6.3 and 6.4 show that a mixture of products is formed by the decomposition of (*R*)- and (*S*)-dihydroxypropylcobalamin. The composition of the mixture depends on whether the cobalamin was photolysed or thermolysed, and on the presence or absence of oxygen.

The product that was usually found was hydroxyacetone, up to 70 % (± 10 %) with respect to cobalamin. The only experiment in which it could not be detected was the anaerobic thermolysis of the *S*-isomer. Hydroxyacetone was identified by its ^{1}H NMR spectrum, and was partially labelled with a single deuterium atom on its methyl group (C3), as shown by the 1 : 1 : 1 triplet at δ 2.12 (2H, J = 2.3 Hz), and the singlet at δ 2.14 (3H) for the unlabelled compound. The proportion of ^{2}H-labelling ranged from 70 to 90 %, estimated from the integrals of these signals. This proportion probably reflects i) the proportion of labelled water in the deuterium oxide used in the experiments (nominally 99 %, but lowered by exposure to air, and by the water included in cobalamin crystals), and ii) the isotope effect for the ketonisation of the enol of hydroxyacetone, which is presumably the initial product of β-elimination (Scheme 6). Heating unlabelled hydroxyacetone at 90 ° for 8 h in D_2O gave less than 5 % of labelled species, so the labelling did not occur by exchange after the hydroxyacetone was formed.

The identity of this compound was confirmed by the formation of its dinitrophenylhydrazone, which had an identical R_f (0.30, silica gel, CH_2Cl_2) to that of an authentic sample. A second spot was also seen on the t.l.c. of the DNP derivatives of the reaction mixture (R_f = 0.24), which has not been identified, either by ^{1}H NMR of the mixture of DNP derivatives, or by comparison with possible compounds. It was thought that it could be 3-hydroxypropanal, but

synthesis of this compound by the periodate oxidation of butane triol[217] showed that this was not the case. Neither the free aldehyde nor its dimer were found in the ^{1}H NMR spectra of the cobalamin decomposition products.

The other firmly identified product was allyl alcohol, formed in up to 75 % yield. It was the only product detected in the anaerobic thermolysis of (*S*)-dihydroxypropylcobalamin, but was found in only 50 % yield in this experiment.

The presence of both hydroxyacetone and allyl alcohol was demonstrated by gas chromatography (section 6.3.2c). Other possible products; glycerol, glyceraldehyde, 3-hydroxypropanal, propane-1,2-diol, propane-1,3-diol, 1-propanol, 2-propanol, acetone, and propanal were all separated on the column used, but were not found in the samples from the reaction mixtures.

Other signals were found in the ^{1}H NMR spectra, particularly in the aerobic experiments. A doublet at δ 3.51 (J = 5 Hz), and a triplet at δ 5.05 (J = 5 Hz) with about half the intensity of the doublet, were found in the anaerobic photolysis of both diastereoisomers. It could be an oxidation product, *e.g.* glyceric acid.

Conducting the experiment at pH 4 did not make any difference to the anaerobic photolysis of the *S*-isomer.

Overall, there were a few differences between *R*- and *S*-isomers. The pattern of products was similar in each case, but the proportions of products varied by up to 25 %. Differences of almost this magnitude were sometimes found between duplicate experiments with the same isomer, and so the observed differences may reflect variations in conditions, such as the concentration of oxygen, or the presence of extraneous reagents. It is therefore not possible to distinguish (*R*)- and (*S*)-dihydroxypropylcobalamin on the basis

of their photolytic and thermolytic behaviour.

In general, the following results were found:

i) Aerobic photolysis gave hydroxyacetone as the main product (70 %) with other (possibly more oxidised) products. Allyl alcohol was not found.

ii) Anaerobic photolysis gave hydroxyacetone and allyl alcohol (ratio 2 : 1), while aerobic thermolysis gave the same products in ratios (6 : 1 (*R*), 7 : 3 (*S*)). Both sets of experiments gave these products in almost quantitative yield, but aerobic thermolysis in the presence of excess of oxygen produced the more oxidised product from (i) as well as a small amount of hydroxyacetone.

iii) Allyl alcohol was the main product of anaerobic thermolysis, being formed in 75 % yield (*R*) and 50 % yield (*S*). For the *R*-isomer, about 20 % hydroxyacetone was also produced, while for the *S*-isomer, no other products were identified.

A rationalisation for these findings is as follows (Scheme 6.4). The possible initial reactions of the cobalamin are a homolysis of the Co-C bond to give a primary alkyl radical, b elimination of hydroxide to give allyl alcohol and hydroxocobalamin, and c concerted β-elimination. Pathways a and c are reversible, since the radicals from a can recombine, and hydridocobalamin may be able to re-add to the double bond. In the presence of oxygen, hydridocobalamin is rapidly oxidised to cobalamin(III), and so the enol ketonises to become hydroxyacetone.

The radical formed by a has several options open to it. Recombination with cobalt(II) is known to be very rapid, approaching diffusion-controlled rates[160]. It can also undergo β-elimination of a hydrogen atom[52] leading to the enol of hydroxyacetone and hydridocobalamin, or β-elimination of a hydroxyl radical c, giving allyl

alcohol and hydroxocobalamin. Again, d may be reversible, whereas e may not be. Oxygen can react with the radical pair to give a peroxy-species[218] which decomposes to give hydroxocobalamin and, possibly, glyceraldehyde or glyceric acid, although these were not possitively identified in the reaction mixtures. Oxygen also oxidises hydridocobalamin to hydroxocobalamin, preventing the reverse reaction of d, allowing the enol to ketalise to hydroxyacetone.

It is difficult to distinguish between a homolytic and a concerted mechanism of β-elimination[52,118]. The cobalt-carbon bond is known to be relatively weak[203], and so readily undergoes homolysis. Recombination of the cage-trapped radical pair, however, is also extremely fast, and so elimination may take place either when the bond is formed or dissociated. The differences observed in the presence of oxygen may indeed be due to the reaction of oxygen with the radical pair, but this does not prove that the eliminations proceed *via* the intermediate radicals.

The results can be explained with reference to Scheme 6.4.

i) Aerobic photolysis led to hydroxyacetone and more oxidised products. The presence of oxidised products suggests a homolytic pathway a; since alkylcobalamins do not react with oxygen in the absence of heat or light. No allyl alcohol was found, which suggests either that the radical pair generated by a is in an excited state which cannot lose a hydroxyl radical, or that e is much slower than d under these conditions. The hydridocobalamin formed in d is oxidised to the unreactive hydroxocobalamin, and hydroxyacetone is formed by ketonisation of the enol resulting from β-elimination.

ii) Anaerobic photolysis led to hydroxyacetone and allyl alcohol in the ratio 2 : 1. Allyl alcohol can be produced by the elimination c of hydroxyl radical to give hydroxocobalamin. A similar loss of

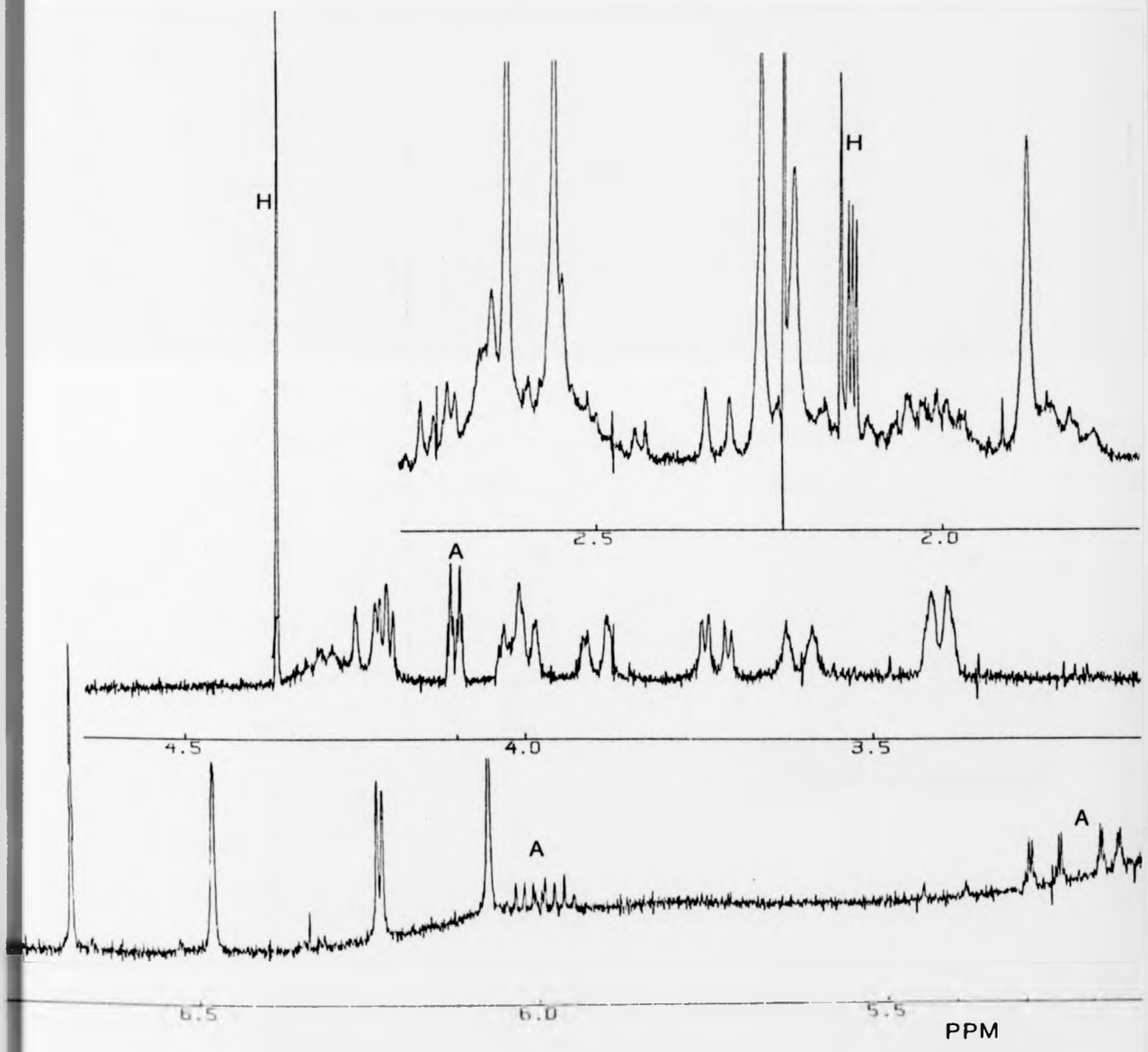
a β-hydroxy group from an alkylcobalamin has not been reported before, although β-hydroxyalkylcobaloximes are known to eliminate water under acidic conditions to give olefins[219]. In the absence of oxygen, the hydridocobalamin formed in d is removed much more slowly, either by reaction with hydroxocobalamin from e to give cobalamin(II), or by reaction with another molecule of hydridocobalamin to give cobalamin(II) and hydrogen. Therefore the ratio of allyl alcohol to hydroxyacetone depends on the relative rates of e and the ketonisation f, and on the rate of removal of hydridocobalamin by one of the above reactions.

iii) Anaerobic thermolysis led to allyl alcohol (50 % for the *S*-isomer, 75 % for the *R*-isomer), with, in addition, about 20 % hydroxyacetone formed from the *R*-isomer. The remaining 50 % of the organic ligand of the *S*-isomer, and 30 % of the *R*-isomer could not be accounted for. The differences between these proportions and those of the anaerobic photolysis can be explained in a number of ways, and as yet, there is no evidence to distinguish the various possibilities. It is possible that concerted eliminations are more important in thermolysis than are radical pathways, and so the product ratios reflect the relative rates of b and c. Alternatively, the radical pair may be formed as in a, but its mode of decomposition may be different. Light-induced homolysis may lead to a different excited state that that produced by heating, and so the relative rates of e and d may be altered. Even if the same species is produced by a, the difference in temperature may alter the rates e and d, and the rate of decomposition of hydridocobalamin, so giving a change in the ratio of products between photolytic and thermolytic decomposition.

iv) Aerobic thermolysis also gave a mixture of allyl alcohol and hydroxyacetone, with hydroxyacetone as the major product. This is to be expected, since the equilibrium in c or d is pulled towards the

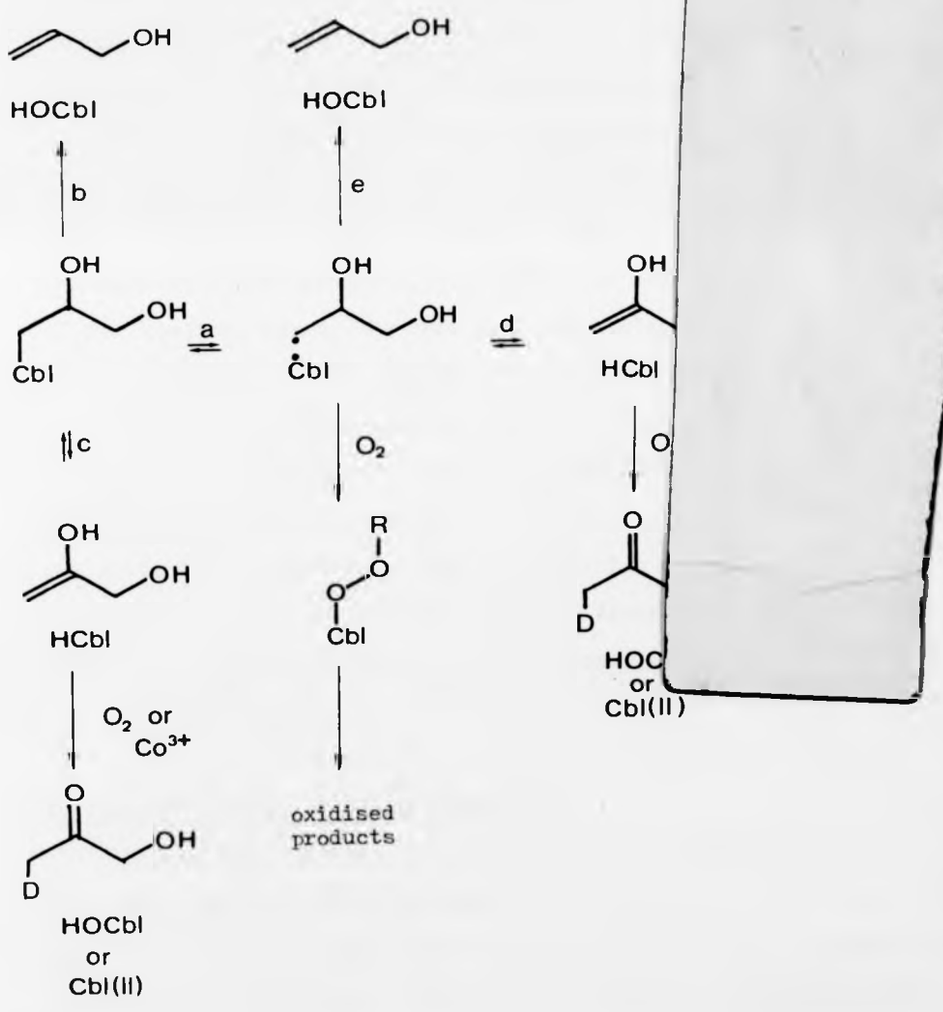
Products of anaerobic photolysis of (S)-dihydroxypropylcobalamin

H = hydroxyacetone
A = allyl alcohol



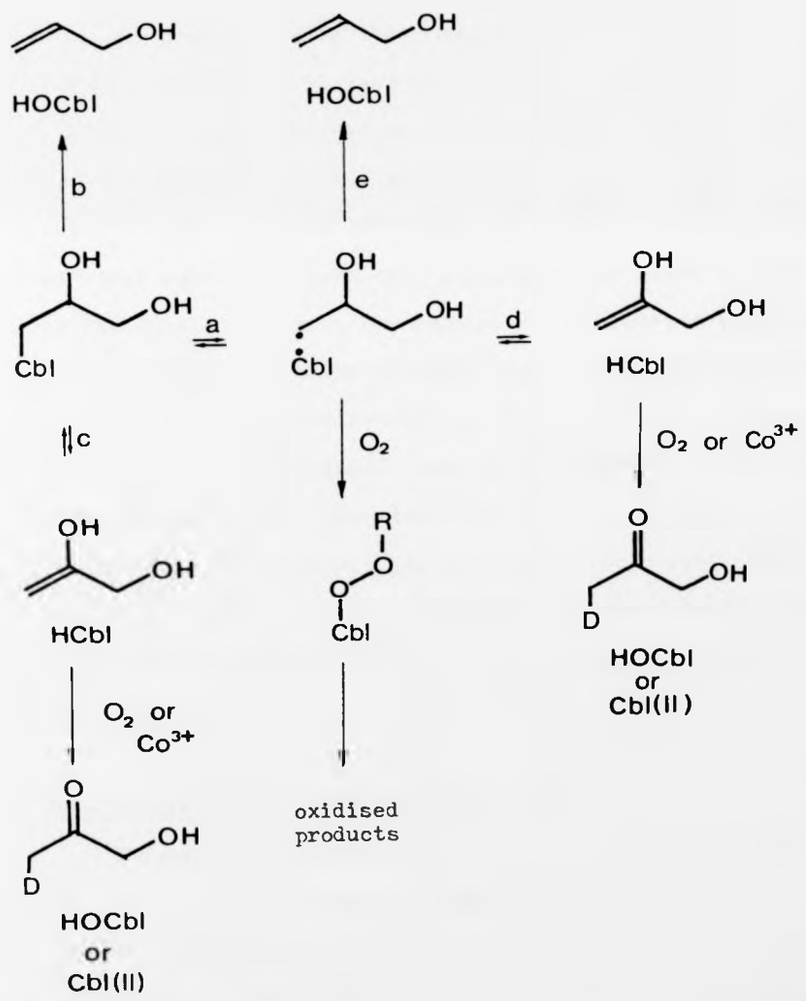
formation of hydroxyacetone by the removal of hydri
oxidation.

Scheme 6.4 Possible decomposition pathways for dihydroxy



formation of hydroxyacetone by the removal of hydridocobalamin by oxidation.

Scheme 6.4 Possible decomposition pathways for dihydroxypropylcobalamins



6.4.4 3,4-Dihydroxybutylcobalamin (Table 6.5).

This compound (the 4*R* + 4*S* mixture) gave butene-3,4-diol in all the experiments, and exclusively in anaerobic photolysis. Two other products with signals in the olefinic region were found in other experiments. One had signals at δ 5.69 (dd, J = 6,4 Hz), and δ 5.53 (d, J = 4 Hz) and was found in aerobic thermolysis and photolysis, possibly in up to 25 % yield with respect to cobalamin. It could not be identified. The other had signals suggesting a terminal double bond (δ 5.24, dd, J = 11, 1.5 Hz, δ 5.33, dd, J = 17, 1.5 Hz, and δ 5.85, m), and was formed by anaerobic thermolysis in < 20 % yield. It was tentatively assigned to be a mixture of *E*- and *Z*-[1-^{2}H]-butene-3,4-diol (see section 6.4.7). No aldehyde signals were seen, but DNP treatment showed a complex mixture of carbonyl-containing products, although these might arise by degradation of the cobalamin during DNP treatment. No butanal was found, which is in agreement with the findings from 4,5-dihydroxybutylcobaloxime.

It seems from the results in Table 6.5 that β-elimination of hydrogen is the most usual mode of decomposition of 3,4-dihydroxybutylcobalamin, but other reactions compete in some cases. Not all the products were identified, so a mechanistic explanation cannot be offered. The possible relationship of these findings to those on 4,5-dihydroxypentyl- and 5,6-dihydroxyhexylcobalamin are discussed below (section 6.4.7).

6.4.5 4,5-Dihydroxypentylcobalamin (Table 6.6)

The mixture of (4*R*)- and (4*S*)-dihydroxypentylcobalamin gave the product of β-elimination, pent-1-ene-4,5-diol, under all the conditions studied, and it was the only product of aerobic thermolysis, and photolysis when oxygen rather than air was used to oxygenate the solution. The product was identified by the synthesis of an authentic

sample by the photolysis of 4,5-dihydroxypentylcobaloxime in neutral solution which is known to give the terminal olefin[106].

Sometimes the integrals of the olefinic signals did not agree with those expected, and the shapes of the peaks differed from those of the authentic compounds (Figure 6.1). It is likely, therefore, that deuterium labelling has occurred on C1, giving evidence of the possibility of re-addition to the double bond (Scheme 6.6) and this is further discussed in section 6.4.7.

A second olefinic product was formed by anaerobic thermolysis and photolysis, and by aerobic photolysis with limited amounts of oxygen. It also appeared to be the major product in the anaerobic photolysis at p^2H 3. It was tentatively identified on the basis of decoupling experiments (Table 6.8) as pent-2-ene-4,5-diol. Attempts were made to synthesise a sample of this compound by epoxidation of penta-1,3-diene, followed by hydrolysis, and by a Wittig reaction of acetaldehyde with the triphenylphosphonium salt of 4-iodomethyl-2,2-dimethyl-1,3-dioxolan, but these were not successful.

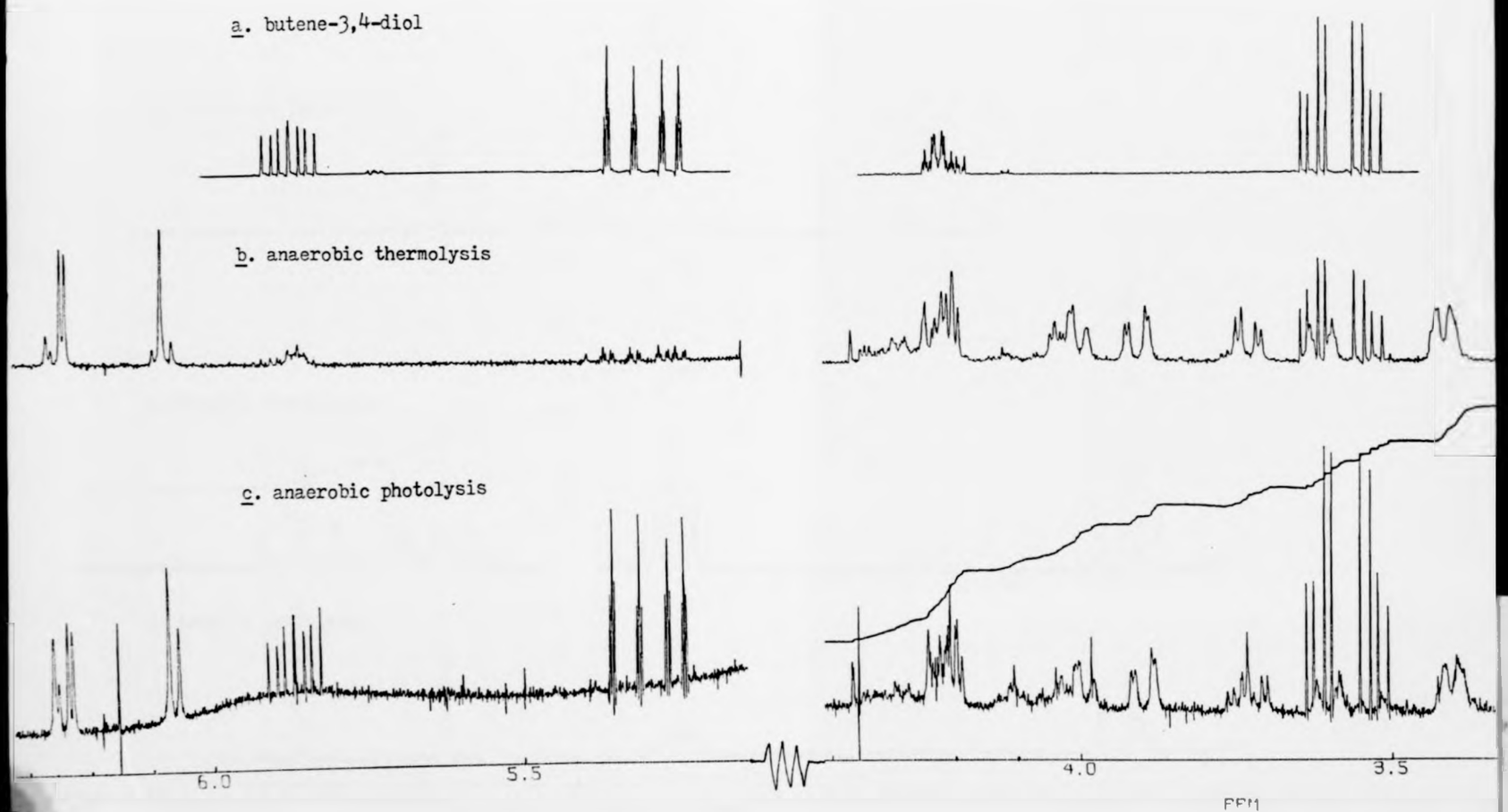
Treatment of the reaction mixtures with DNP gave no evidence of pentanal, by comparison of the t.l.c. of the derivatives with an authentic sample. Other carbonyl-containing products were found, but could not be identified.

An explanation is offered for the formation of a product with an internal double bond, and it is discussed in section 6.4.7, with the results of 5,6-dihydroxyhexylcobalamin which show marked similarities.

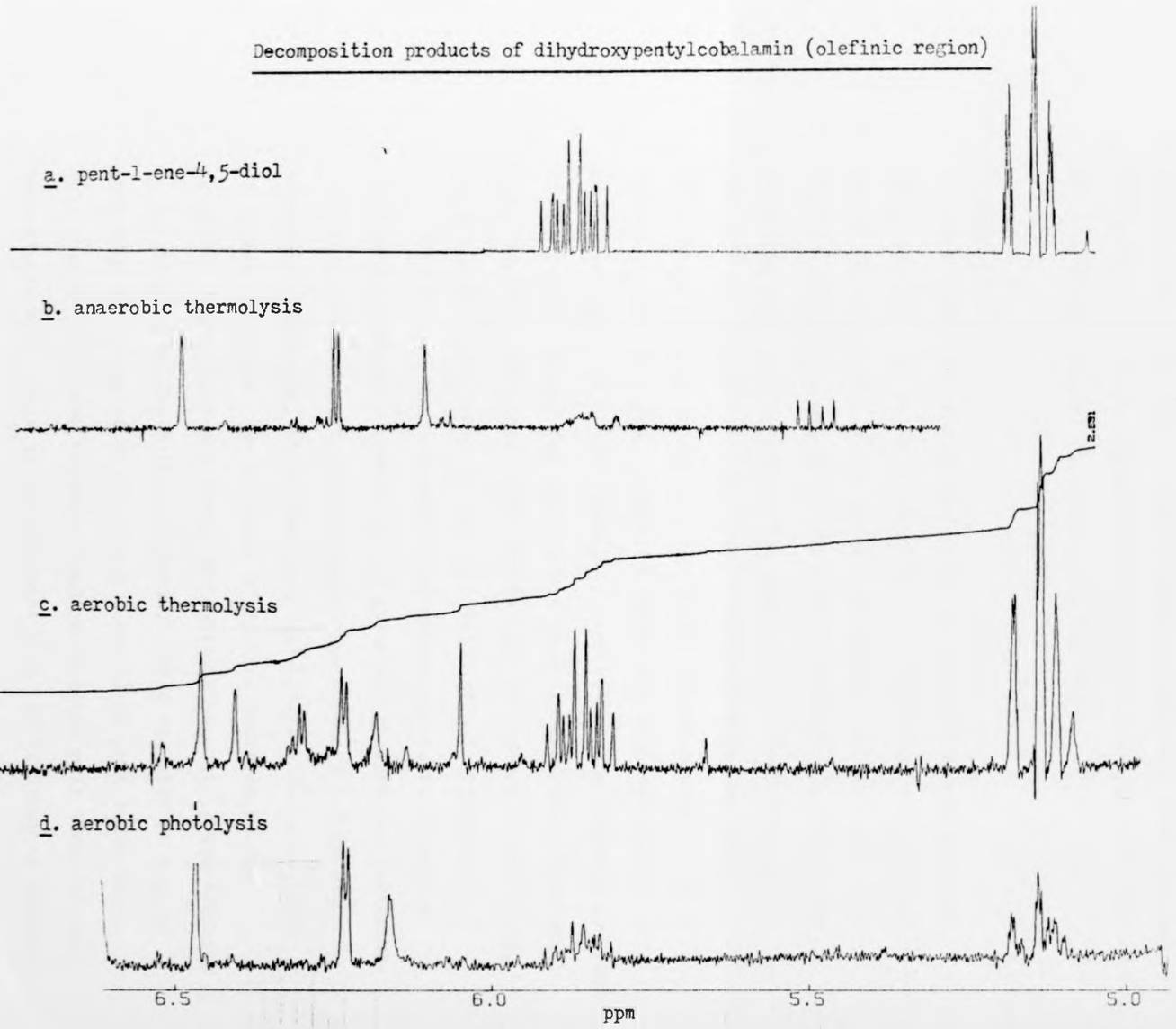
6.4.6 5,6-Dihydroxyhexylcobalamin Table 6.7

The decomposition of the (5*R* + 5*S*) mixture of these cobalamins led to a similar pattern of products as the decomposition of the

Decomposition products of dihydroxybutylcobalamin



Decomposition products of dihydroxypentylcobalamin (olefinic region)



dihydroxypentylcobalamins. That is, under aerobic conditions, the β-elimination product of hex-1-ene-5,6-diol was produced in quantitative yield, while under anaerobic conditions much less was produced, and signals suggestive of an internal olefin were seen. No evidence of hexanal or hexan-2-one was found.

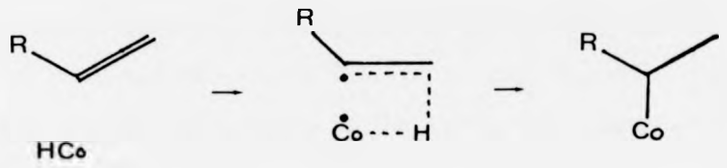
6.4.7 Discussion

The formation of a product with an internal double bond can be rationalised if it is assumed that β-elimination to give the olefin is reversible in the absence of oxygen (Scheme 6.6). The hydridocobalamin formed by β-elimination (either by a radical or concerted pathway) can add to the other end of the double bond, possibly *via* a π-complex, to give a secondary alkylcobalamin. This, being unstable under the conditions of the reaction, again undergoes β-elimination, either to reform the terminal olefin, or to give an internal olefin (probably *trans*, since a coupling constant of 15 Hz was measured for the product from dihydroxypentylcobalamin). Under aerobic conditions, hydridocobalamin is removed by oxidation, and a quantitative yield of the terminal olefin is produced.

Evidence for the re-addition of hydridocobalamin to double bonds under anaerobic conditions is given by the apparent deuterium-labelling of C1 of pent-1-ene-4,5-diol and butene-3,4-diol under anaerobic conditions, while they were unlabelled under conditions of excess of oxygen. No evidence of further addition to the secondary double bond was found, possibly because it is too hindered to be attacked by the bulky cobalamin. There was also no sign of deuterium labelling of C2 of butene-3,4-diol or pent-1-ene-diol. This suggests that the terminal bond is attacked only from the more substituted end. This is in agreement with the findings of Halpern

and Wong[216] who suggest a diradical or radical-like transition state for this type of addition (Equation 6.3).

Equation 6.3

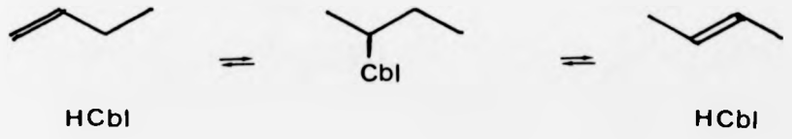


The secondary radical is more stable than the primary one, so the cobalt adds to C2 rather than to C1.

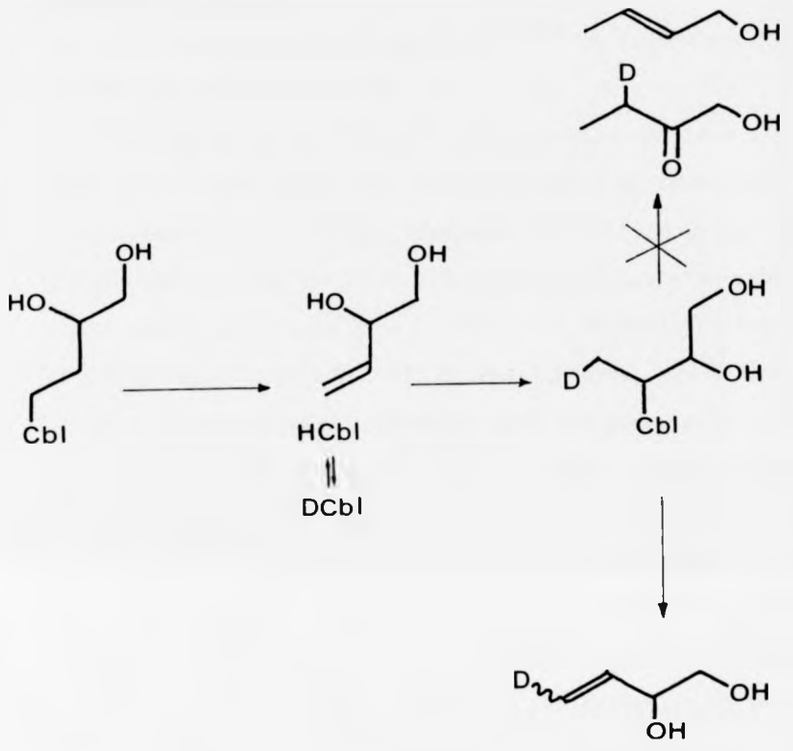
This hypothesis explains the results from dihydroxypentyl- and dihydroxyhexylcobalamins, but not those from dihydroxybutylcobalamin, since, for instance, butene-3,4-diol was the only product of anaerobic photolysis. If a similar mechanism was operating, products such as but-2-en-1-ol and 1-hydroxybutan-2-one might be expected, by analogy with dihydroxypropylcobalamins. No such products were found in any of the reaction mixtures, although other products were present which suggest that the addition to the double bond is reversible. It is possible that the hydroxyl group on C3 of butene-3,4-diol prevents that addition of hydridocobalamin to the double bond at C2, for steric or electronic reasons, or that if addition does take place, β-elimination from C1 is favoured, and so the original terminal olefin is formed. If addition did take place in this way, the terminal carbon atom might sometimes pick up a deuterium atom

from the solvent (or from the cobalt-deuteride species) to give the secondary alkylcobalamin in Scheme 6.5, which undergoes β-elimination to give a mixture of *E*- and *Z*-[1-^{2}H]-butene-3,4-diol. The observed signals from anaerobic thermolysis support the formation of such compounds in about 20 % total yield.

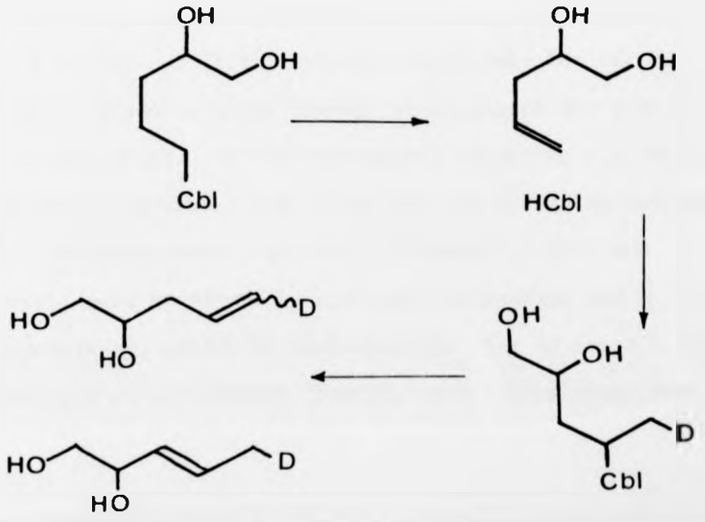
The formation of these labelled compounds is unexpected, as addition of cobalt-hydride species to double bonds would be expected to go through a four-centre transition state (Equation 6.2b), with direct transfer of hydrogen to the olefin, and therefore, no labelling should occur. If, however, under the reaction conditions the cobalt-hydride exchanges with deuterium from the solvent, then the labelling can be explained. At the pH values in most of the reactions (pH 6 - 8) the concentration of the cobalt hydride species should be very low indeed (pKa ∿ 1), but the observed products suggest that it is present as the reaction of cobalamin(I) with unactivated olefins has not been observed. Hydridocobalamin reacts with olefins in acidic solution to give secondary alkylcobalamins[118], as observed here.



Scheme 6.5 Decomposition of dihydroxybutylcobalamin



Scheme 6.6 Decomposition of dihydroxypentylcobalamin



6.4.8 Cobaloxime studies

The findings by Golding *et al.*[31,56] on dihydroxyalkylcobaloximes are summarised here.

Photolysis of 4,5-dihydroxypentylcobaloxime in 0.1 M acetic acid (pH 3) under anaerobic conditions gave pentanal (10 - 15 %), pent-1-ene-4,5-diol (30 %), pentane-1,2-diol (20 %) and decane-1,2,9,10-tetraol (20 %). 5,6-Dihydroxyhexylcobaloxime photolysed under these conditions gave a mixture of hexanal and hexan-2-one (20 % in total, ratio 1 : 4), hexane-1,2-diol, and hexane-5,6-diol. At pH 7, both the above compounds gave the product of β-elimination as the only identified product (30 % in the case of 4,5-dihydroxypentylcobaloxime, yield unrecorded for 5,6-dihydroxyhexylcobaloxime).

The photolysis of 3,4-dihydroxybutylcobaloxime did not give butanal or butanone under any conditions, and 10,11-dihydroxyundecylcobaloxime also did not give any carbonyl-containing products.

Deuterium-labelling showed that the formation of pentanal and hexan-2-one was by a [1,5]-hydrogen shift, and hexanal by a (less favourable) [1,6]-shift, followed by acid-catalysed dehydration. The absence of butanal or pentan-2-one showed that [1,4]-hydrogen transfer is even less favourable, and the absence of carbonyl products from 10,11-dihydroxyundecylcobaloxime showed that, as expected, a [1,10]- or [1,11]-hydrogen shift does not occur (Scheme 6.1).

The other products arose through reactions of the radicals produced by homolysis. In the experiments involving 4,5-dihydroxypentylcobaloxime, decane-1,2,9,10-tetraol was formed by the coupling of two 4,5-dihydroxypentyl radicals. Pentane-1,2-diol and pent-1-ene-4,5-diol could be formed by hydrogen abstraction and by hydrogen loss, respectively, either by disproportionation of two 4,5-dihydroxypentyl radicals or by hydrogen transfer to and from other reagents.

In acid solution, cobaloxime(II) is kinetically labile, and decomposes to dimethylglyoxime and aquated cobalt(II) ion. At pH 7, bisaquo-cobaloxime(II) is found, produced by the reaction of hydridocobaloxime to give hydrogen and cobaloxime(II).

The differences between the cobaloxime and the cobalamin experiments seem to be due to i) the ability of hydridocobalamin to react further with the olefins formed by β-elimination, and ii) the instability of cobaloxime(II) in acid solution, which prevents the recombination of the radical pair generated by photolysis, thus releasing the alkyl radical to undergo inter- or intramolecular hydrogen transfer, or other radical reactions.

6.4.9 Summary and projected work

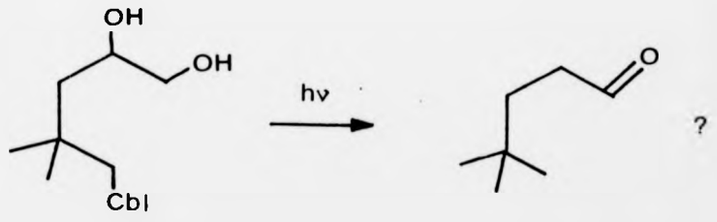
The cobalamin model system described here does not, therefore, mimic the vitamin-B_{12}-dependent reaction of diol dehydratase in the way that the cobaloxime systems do. The cobaloximes model the regiospecific removal of a hydrogen atom from C1 of a 1,2-diol, and no evidence of such transfer was found with the cobalamins. These studies are of value, nevertheless, in that they show that the cobalt(II) species formed by homolysis of the cobalt-carbon bond can abstract a hydrogen atom from the alkyl radical, to give an olefin, and that this is the most likely reaction unless the cobalt radical is removed in some other way (for instance by oxidation, or by dissociation of the ligands (cobaloximes)). It has also been shown that the hydridocobalamin formed in this reaction can add to the other end of the double bond so formed, in acidic or in neutral solution, and so give products of β-elimination of the secondary alkylcobalamin.

It seems clear, therefore, that if the cobalt(II) species is

present, the hydrogen abstraction from C1 of the diol does not take place. This suggests that the radical formed from the [1,5]-hydrogen transfer must rearrange without participation from the cobalt, a finding which is in agreement with the 'bound-radical hypothesis' proposed by Golding[88], Abeles[66], Finke[53] *etc.*, in which the reactive substrate-derived radical in the enzymic reaction does not combine with cobalt, but is prevented from escaping to damage other systems, by the protein, which may also assist the rearrangement, for instance by protonation[93] or deprotonation[53] of one of the hydroxyl groups. This hypothetical mechanism is summarised in Scheme 6.8.

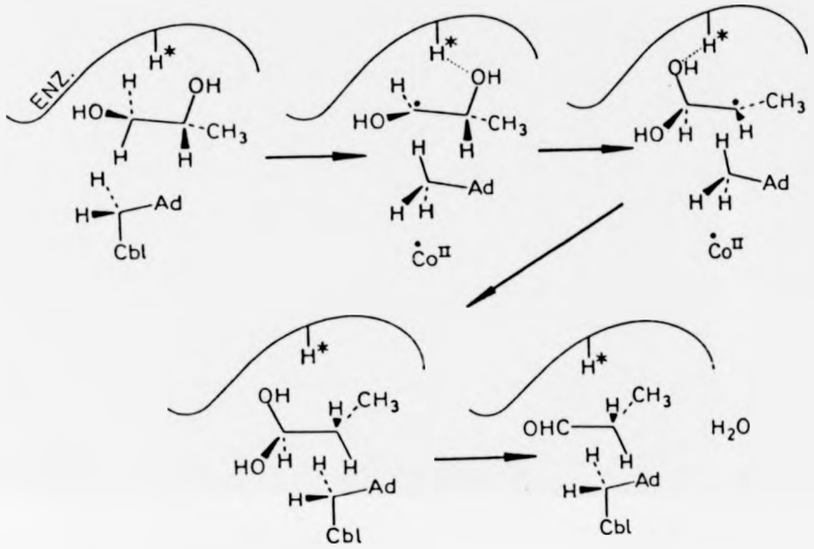
To inhibit β-elimination, it was decided to synthesise 4,5-dihydroxy-2,2-dimethylpentylcobalamin (60). This was not isolated as a pure substance, because of the difficulty of preparing the alkylating agent (Chapter 2) but the ^{1}H NMR spectrum showed it to be present in the mixture of cobalamins. Anaerobic photolysis of the mixture, in [$^{2}H_4$]-acetic acid (0.1 M) gave a sharp singlet at δ 1.08, just possibly corresponding to a tertiary butyl group (Scheme 6.7). There were also small aldehyde signals at δ 9.2 and 9.3, but these results are very tentative. Work is proceeding to isolate and study the pure cobalamin.

Scheme 6.7



(60)

Scheme 6.8 The protein-bound-radical mechanism[89]



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